THE EPIDEMIOLOGY AND MOLECULAR CHARACTERIZATION OF COLORECTAL CANCER IN EASTERN NEWFOUNDLAND

FIONA K. CURTIS

NOTE TO USERS

The Epidemiology and Molecular Characterization of Colorectal Cancer in Eastern Newfoundland

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ABSTRACT

Background: Hereditary Non-Polyposis Colon Cancer (HNPCC) is a dominantly inherited disorder caused by germ-line defects of mismatch repair (MMR) genes. HNPCC can be diagnosed using clinical and genetic criteria. Few population-based studies have been undertaken to determine the genetic basis of CRC. The purpose of this pilot project was to determine the proportion of hereditary vs. sporadic CRC cases on the Avalon Peninsula (AP) of Newfoundland and to determine the genetic basis of disease in hereditary cases.

Methods: The population studied was identified through the Newfoundland Cancer Treatment and Research Foundation (NCTRF) registry, the meditech system (a hospital reporting tool), and pathology reports. One hundred and sixty-eight potential probands were identified, diagnosed in either 1997 or 1998, between the ages of 20-69, and residing on the AP. Probands, or an appropriate next of kin if a proband was deceased, were interviewed to determine family history of cancer. Contact was made with 158 eligible participants of which one hundred and six (67%) agreed to participate in the study. The final number of study subjects was 79, which was 74.5% of those who agreed to participate. The NCTRF registry and a review of medical charts were used for the collection of baseline characteristics which included demographic and clinical data.

Probands were classified by their family history using the Amsterdam Criteria (AC), Amsterdam II Criteria, and several other criteria to identify familial risk of HNPCC. Microsatellite analysis (n= 74) and immunohistochemistry analysis (n= 71) for

hMLH1, hMSH2, and hMSH6 was completed on proband's normal and tumour DNA. We also examined the diagnostic utility of microsatellite analysis and of protein expression to identify High Risk (HR) families, in comparison to Low Risk (LR) families. **Results:** Twenty-two (28.2%) families were considered HR with 6 families fulfilling the AC I, 1 family fulfilling the AC II, and 15 families fulfilling our Age and Cancer Modified AC (youngest cancer $\leq$ 60 rather than 50). Twenty-two (28.2%) families were Intermediate Risk (IR), and 34 (43.6%) families were (LR) for HNPCC. However 13 of the LR families had an uninformative family structure. All Amsterdam Criteria families had previously been referred to the Newfoundland Medical Genetics program.

Sixteen cases (21.6%) had high frequency microsatellite instability (MSI-H) tumours. Sixteen cases (22.5%) had absence of protein expression for at least one of three proteins. For the HR group 6 (31.6%) had MSI-H tumours and all showed absence of protein expression. Fourteen (68.4%) of the HR group had microsatellite stable (MSS) tumours of which 13 (92.9%) expressed all the proteins. For the IR group 4 (22.2%) had MSI-H tumours and all showed absence of protein expression. Fourteen (77.8%) of the IR group had MSS tumours and all expressed the proteins. For the LR group 4 (12.5%) had MSI-H tumours and 3 (9.4%) of these showed absence of protein expression. Twenty-eight (87.5%) of the LR group had MSS tumours of which 27 (96.4%) expressed all proteins.

The sensitivity of the laboratory methods used in predicting cases that are high risk for HNPCC was 35.0% and the specificity of the methods in predicting cases that do not have HNPCC was 78.9%.

Conclusions: We conclude that in the Eastern Newfoundland population the risk for HNPCC is high. Twenty-eight percent of families with CRC are at high risk for HNPCC. Furthermore the prevalence of MSS tumours in the HR group was high. It is likely that novel genes predisposing to HNPCC occur in the Newfoundland population. The utility of testing for MSI and MMR protein expression for the diagnosis of HNPCC in this population was poor.

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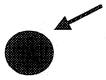
Chapter 3- RESULTS

CANCER SITE ABBREVIATIONS

bl	- Bladder
bo	- Bone
br	- Breast
CRC	- Colorectal
cx	- Cervix
ear	- Ear
eg	- Epigastric
en	- Endometrium
gb	- Gallbladder
hk	- Hodgkin's Disease
ki	- Kidney
li	- Liver
lip	- Lip
lk	- Leukemia
lu	- Lung
lym	- Lymphoma
mel	- Melanoma
mm	- Multiple Myeloma
mo	- mouth
oes	- Oesophagus
ov	- Ovary
pa	- Pancreas
pr	- Prostate
sk	- Skin
te	- Testis
th	- Throat
to	- Tounge
ut	- Uterus

LIST OF PEDIGREE SYMBOLS

Affected Proband male

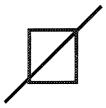


Affected Proband female

HNPCC cancer

Non-HNPCC cancer

Unaffected Siblings (male or female)



Deceased

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CHAPTER 1: INTRODUCTION

This pilot project examines the family risk for Hereditary Non-Polyposis Colon Cancer (HNPCC) of probands identified in Eastern Newfoundland during 2 years. It was developed by two graduate students, one in Molecular Genetics and the other in Clinical Epidemiology. Both projects and their outcomes rely on the existence of the other. This study is a pilot project for the Newfoundland component of the Collaborative Interdisciplinary Health Research Team (IHRT) study, between Memorial University and Mt. Sinai Hospital, which includes development of a Newfoundland Familial Colorectal Cancer Registry (NFCCR). The development of a specialized colorectal cancer (CRC) population-based registry could lead to estimates of the true incidence of HNPCC within the population of Newfoundland. Such registries specific to CRC exist in other populations.

The Newfoundland Cancer Treatment and Research Foundation (NCTRF) is a provincial organization responsible for the delivery of cancer services including prevention, treatment, support, and research to the people of Newfoundland and Labrador. It contains a detailed provincial tumour registry. The registry collects data on all new cancer diagnoses within the province. It contributes to the Canadian Cancer Statistics providing detailed information concerning the incidence and mortality of cancer within the province. However it does not contain family risk information.

Screening has been shown to reduce the occurrence of CRC in HNPCC (Jarvinen et al, 1995). By identifying families affected with HNPCC, and offering screening programs to those at risk, earlier diagnosis and treatment is possible with better prognosis.

It is important to determine the incidence of HNPCC in Newfoundland so that the need for appropriate resources for management will be put into place.

1.2 LITERATURE REVIEW

The following factors have all led to improved prevention and treatment options for CRC: the early recognition of cancer clusters; the identification of HNPCC kindreds; the search for genes and mutations in kindreds; the use of known mutations to develop new and better techniques for mutation detection; and the use of mutations to find and screen family members at risk.

HNPCC occurs worldwide as documented in Australia (Scott et al, 2001), Canada (Bapat et al, 1999), Denmark (Jager et al, 1997), Finland (Moisio et al, 1996), France (Wang et al, 1999), Germany (Lamberti et al, 1999) Great Britain (Dunlop et al, 1997), Italy (Ponz de Leon et al, 1999), Japan (Miyaki et al, 1995), Korea (Han et al, 1996), Netherlands (Wijnen et al, 1998a), Spain (Godino et al, 2001), Sweden (Liu et al, 1998), Switzerland (Buerstedde et al, 1995), United States (Weber et al, 1997), and others. Finland has reported two out of four of the world's known founder mutations to date that are responsible for the majority of putative HNPCC (51%) and HNPCC (68%) families in Finland (Nystrom-Lahti et al, 1996). This finding was possible not only because of an efficient cancer registry and population registry, but also because of its unique population. Other founder mutations have been reported in both the Danish (Jager et al, 1997), Newfoundland (Froggatt et al, 1999), and more recently American (Wagner et al, 2003) populations.

Studies to determine the population prevalence of genotypes and phenotypes are influenced by lack of consistency in methods and definitions. Efforts to collaborate have been facilitated by several groups. The international collaborative group on HNPCC (ICG-HNPCC) was initially formed by experts in the field in the early 1990's (Vasen et al, 1991). This group plays a pivotal role in HNPCC education, development of registries, and continuing international research. The National Cancer Institute (NCI) is an establishment in the United States. NCI's mandate is to support cancer research and to develop and implement the findings of the research. Currently there are 6 international sites participating in the Co-operative Familial Colorectal Cancer Registries sponsored by the NCI (Cotterchio et al, 2000).

1.21 Genetic Epidemiology

A new discipline has emerged that combines the methodologies of both Epidemiology and Genetics. It aims to describe and determine disease distribution in populations and families. It draws on a number of sources including newly developing registries and monitors to help understand etiologic heterogeneity, genetic-environmental interactions, and natural history of diseases (Khoury et al, 1986). Efforts are made to collect data on disease status, risk factors, family history, and to collect DNA. Analysis of a combination of these sources will lead to intervention and prevention, and will impact disease trends in populations (Vogel et al, 1979; Khoury et al, 1986). The observation of patterns for familial aggregation can be a starting point of genetic–epidemiological studies (Khoury et al 1986; Wickramartrie, 1995). This provides an opportunity to study modifying factors in kindreds that possess the same mutation.

Mendelian disorders are single-gene traits that segregate within families, and should occur in fixed proportions among the offspring. They are often characterised by their pattern of inheritance in families. There are 4 basic patterns of single gene inheritance; autosomal dominant, autosomal recessive, X-linked dominant, X-linked recessive (Thompson, 1991). Management of these disorders assumes that interpretation of the mode of inheritance is correct, and that the affected individual is classified correctly and representative of the condition (Watson and Lynch, 1993). Identifying specific genetic mutations improves the management. However, numerous obstacles can complicate finding these mutations and thus complicate the management of Mendelian disorders. These include incomplete penetrance, small family size, new mutations, variable expressivity of the trait, and variable age of onset. Environment, gene-environment, and gene-gene interactions potentially influence gene expression. Although individual genetic disorders are rare in the general population, in combination they are an important cost to society as they lead to serious morbidity and mortality. Large studies are needed to study these rare genetic traits.

Cancer is a relatively common occurrence in the general population and a serious cause of morbidity and mortality in the world. The goal of genetic epidemiology research in cancer is to ultimately lead to prevention and control. Providing surveillance and prevention programs for identified groups with an increased risk of developing cancer could impact the cancer burden. There are twenty hereditary or familial cancer syndromes in which the risk of cancer is high (Fearon, 1997).

1.22 Cancer

Cancer is a universal term used to describe a variety of diseases that share a common feature of uncontrolled cellular growth (Vogelstein and Kinzler, 2002). Cancer is the principal cause of premature death in Canada. In 2002, it was estimated that 136,900 Canadians were diagnosed with cancer and 66,200 died of cancer. Although cancer may develop at any age, 25% of new cases occur in those 60-69 and 44% of new cases occur above age 69. Therefore a person's risk of developing a cancer increases with age. It is estimated that approximately 38% of women and 41% of men will develop a cancer in their lifetime (National Cancer Institute of Canada, 2002).

Early diagnosis and early treatment of cancer are imperative to survival. Therefore an important aim of research is to identify individuals at increased risk for cancer before development of cancer. Risk factors influencing the development of cancer include age, diet, level of physical activity, smoking, alcohol intake, and family history. Modifications to lifestyle habits may decrease the risk of developing a cancer. Protective factors include adding fibre and vegetables to the diet (Canadian Cancer Society, 2003). Research indicates that NSAIDS and hormone replacement therapy appear to have a protective effect (Potter et al, 1993).

The processes involved in the pathogenesis of cancer are unclear and may in fact include a combination of gene-gene interactions, gene-environment interactions, or single gene mutations. It is believed that progression to cancer is a multistep process that involves multiple mutations. These mutations can occur in both somatic and germline cells. Oncogenes, tumor suppressor genes and mismatch repair genes are three types of genes that have been implicated in this multistep process. Normally these genes act

simultaneously to control and optimise cellular growth. Mutations arising in these genes begin the multistep process that leads cells to malignant growth (Vogelstein and Kinzler, 2002). While non-genetic factors are suspected to be responsible for the majority of all cancers (sporadic cancers) it is estimated that potentially 0.1%-10% of all cancers has an inherited component (Fearon, 1997; Vogelstein and Kinzler, 2002). Much of the current knowledge about the pathogenesis of cancer has been gained through research in colorectal cancer (Fearon, 1995).

1.23 Colorectal Cancer

Colorectal cancer (CRC) is one of numerous cancers that affect the digestive system, the majority of which occurs in the sigmoid colon and rectum (Canadian Cancer Society, 2003). In Canada, it is the 3rd most common cancer occurring in both men and women, and the second most common cause of mortality due to cancer. It is responsible for 10% of all cancer deaths. Clinical outcomes are related to stage at diagnosis. In 2002, five year survival rates of 59% and 56% were reported for females and males respectively. Fifty-three percent of all new cases occur after age 70 therefore CRC is generally associated with an elderly population. Incidence rates in men and women appear almost equal with estimation that 6.3% of women and 6.7% of men will develop CRC in their lifetime (National Cancer Institute of Canada, 2002). Like any cancer, CRC is a major economic and social problem for patients and their families.

In Canada a declining incidence and mortality rate of CRC has been observed over the past decade and a half and may be explained by changes in lifestyle habits and casual screening (National Cancer Institute of Canada, 2002). Contrary to this, during the

period of 1996-1998, CRC was the most common occurring cancer (12-13% of all cancer diagnoses within that period) seen in both sexes within the Newfoundland population; it had increased 14% from previous years. CRC occurrence was second to breast cancer in females and prostate cancer in males (H Bliss Murphy Cancer Center, 2000).

CRC is recognised as a preventable disease if caught at an early stage. However, because adenomas and early stage CRC are often symptom free the prevention is complicated (Toribara et al, 1995; Thiis-Evensen et al, 1999). Symptoms of CRC include changes in bowel habits, blood in stools, changes in stools, abdominal discomfort, weight loss, and tiredness (Canadian Cancer Society, 1993). Screening is the process of testing people without symptoms to identify early signs of disease. A variety of screening methods have been shown to reduce the incidence of CRC (Winawer et al, 1993; Thiis-Evensen et al, 1999). Barium enema, fecal occult blood test, flexible sigmoidoscopy and colonoscopy are often used alone or together as screening and diagnostic tools.

Colonoscopic polypectomy is a procedure carried out for the removal of polyps. The removal of these polyps is common practice for the prevention of CRC (Winawer et al, 1993). It would be unethical and not feasible to determine the legitimacy of this practice through randomised controlled trials. Instead, observations were made from studies such as The National Polyp Study (O' Brien et al, 1990; Winawer et al, 1992; Winawer et al, 1993). Subjects were randomised into two groups: those screened at 3 years; or those screened at both 1 and 3 years. Follow-up of these subjects was then compared to retrospective data of individuals who refused colonoscopic polypectomy after polyps were found on initial screening and who were followed for an average of 9 years. A major conclusion of the study was that colonoscopic polypectomy resulted in a

lower than expected incidence of CRC compared to the observed incidence of CRC in those who refused colonoscopic polypectomy (Winawer et al, 1993).

The aim of any screening program is to improve the general health of the population by reduction in morbidity and mortality rates. In order to accomplish this task the screening program needs to be feasible, economical and benefits must outweigh the risks. There is no population screening for CRC in Canada, although screening for CRC is recommended beginning at age 50 (Canadian Cancer Society, 2003). Diagnosis of CRC is usually made when symptoms are evident or sometimes by the presence of asymptomatic iron deficiency. Standard therapy for CRC is surgical resection and this is tailored around the presentation at the time of diagnosis. Chemotherapy and radiation are utilised as neoadjuvant and adjuvant treatment (Canadian Cancer Society, 2003).

Although the etiology of CRC remains uncertain, a number of environmental, pre-existing medical conditions and genetic factors are involved. Epidemiological studies demonstrate that environmental factors play a role in the development of CRC. Diets high in fat and low in fibre are implicated in the pathogenesis of CRC (Giovannucci and Willett, 1994; Winawer et al, 1997). The best known example of this is the study of Japanese immigrants to the United States. Even though a very low incidence of CRC was observed in Japan at the time, this dramatically increased in Japanese immigrants (Haenszel et al, 1968). Also more recently, the incidence of CRC in Japan has increased and the incidence of stomach cancer has decreased possibly due to adaptation of the western influences in the diet (Lee, 1976).

Other risk factors for CRC include the inflammatory bowel diseases (IBD), Crohn's disease and ulcerative colitis (UC) (Potter et al, 1999). The lifetime risk is

greater for UC than for Crohn's disease and the development of cancer is dependent upon the site, extent, severity and age of onset of the disease. The risk is 10% for UC (Lennard-Jones, 1996), CRC risk begins to increase at 8-10 years after IBD diagnosis (Levin B, 1995). In Canada more than 150,000 people have IBD and some have a 10% increased lifetime risk of developing CRC (Crohn's and Colitis Foundation of Canada, 2003)

CRC usually develops from an adenoma (commonly known as a polyp) on the inner lining of the gastrointestinal tract. Adenomas are quite common in the general population and most people with adenomas do not go on to develop a cancer, even though adenomas are a risk factor (Spencer et al, 1984; Eide et al, 1986; Lotfi et al, 1986; Atkin et al, 1992). An adenoma begins as a benign tumour that may undergo malignant transformation. Abnormal cells (dysplasia) arising within these adenomas will eventually give rise to adenocarcinomas (O'Brien et al, 1990). Muto et al (1975) proposed the adenoma-carcinoma sequence theory. Over the years this theory has been supported and developed through observational and biochemical studies (Stryker et al, 1987; Williams et al, 1990; Winawer et al, 1993). This process of adenoma to carcinoma development occurs over time, usually decades, and involves multiple genes.

Fearon and Vogelstein (1990) developed a model that has improved the understanding of the molecular genetics of sporadic CRC. This model states that seven genetic changes have to take place in order for the development of a cancer. These changes are characterised by major chromosomal alterations and somatic mutations. Mutations of the Adenomatous Polyposis Coli (APC), p53, and K-ras-2 genes, and loss of

heterozygosity (LOH) commonly but not invariably on chromosomes 5q, 8p, 17p, 18q, and 22q occur during the adenoma-carcinoma sequence (Vogelstein and Kinzler, 2002).

While the majority of CRC is sporadic in nature, genetic factors are believed to contribute to 15% of all CRC (Cannon-Albright et al, 1988; Houlston et al, 1992). An increased risk of CRC has been observed in first-degree relatives of those affected (Ponz de Leon et al, 1987; Fuchs et al, 1994; Slattery et al, 1994; Negri et al, 1998) and, although minor, there is also an increased risk in twins (Lichtenstein et al, 2000). Several inherited predispositions to CRC have been described. There are two main forms of hereditary CRC: Hereditary Non-polyposis Colon Cancer (HNPCC), and Familial Adenomatous Polyposis (FAP). Investigations into these syndromes have not only improved the understanding of hereditary and sporadic CRC but also of other cancer types (Fearon et al, 1990). HNPCC is the number one risk factor for CRC (Mecklin, 1987).

1.24 HNPCC

HNPCC, as its name implies, is characterised by CRC with a demonstrable lack of polyposis (multiple polyposis). Clinical descriptions of HNPCC include: a younger age of presentation than seen in the general population with an average age of onset of 44, proximal location (right-sided cancers), extracolonic cancers, synchronous, metachronous, multiple, and mucinous cancers. It is an autosomal dominantly inherited syndrome, therefore offspring of affected individuals have a 50% chance of inheriting the mutation. (Vasen et al, 1990; Mecklin et al, 1991; Watson et al, 1993; Lynch et al, 1997).

HNPCC is reported as responsible for anywhere from 0.5-13% of all CRC (Vasen et al, 1991; Aaltonen et al. 1994a; Brassett et al, 1996; Bellacosa et al, 1996; Lynch et al, 1996a; Ponz de Leon, 1996; Evans et al 1997; Lynch et al, 1997; Lynch and de la Chapelle, 1999; Moore and Cowled, 1999; Peel et al, 2000). It has been difficult to estimate the true incidence of HNPCC, however Lynch et al (1996b) suggest that it is responsible for 2-4% of all western CRC. The difficulty stems from an ever changing definition of HNPCC because of the clinical and genetic heterogeneous nature of the condition. Currently HNPCC is identified by multiple methods. Initially, the syndrome was defined clinically, then attempts were made to define it pathologically and now it is possible to identify HNPCC at a molecular level. The optimal diagnostic method would be a molecular diagnosis. However, currently this is expensive, time-consuming, and inconclusive due to the heterogeneity of mutations identified and the inability to detect all mutations. Therefore until improvements are made in laboratory studies, the gold standard for HNPCC diagnosis will be by evaluating family history by standardised clinical criteria.

The earliest description of HNPCC was in 1913 by a pathologist Dr. Alfred S. Warthin (Warthin, 1913). This description was of the occurrence of cancers in a family now known as "Family G" (Lynch et al, 1971). HNPCC was first labelled "cancer family syndrome" in 1971 by Henry T. Lynch. Lynch was instrumental in describing the clinical manifestations of this disease and is still an important researcher in HNPCC (Lynch et al, 1971; Lynch et al, 1996a; Lynch et al, 1999). In 1984 HNPCC was divided into the two subcategories Lynch syndrome I (absence of extracolonic malignancies) and Lynch syndrome II (presence of extracolonic malignancies) (Boland et al, 1984; Lynch et al

1985). It is apparent now that there is no genetic basis for this classification (Nystrom-Lahti et al, 1994).

Although HNPCC is uncommon, it represents a group of individuals with an increased risk of developing cancer who might benefit from strategic surveillance and prevention. Screening in other hereditary syndromes has been shown to be economically favourable to health care systems and families, by reducing psychosocial costs to families, and resulting in preferred patient outcomes (Green, thesis 1995). Clinical screening is usually offered to high risk individuals who have been identified by pedigree analysis or predictive genetic testing. Colonoscopy, starting 5 years before the earliest diagnosis of CRC in the family, at one to five year intervals has been recommended by several groups (Mecklin et al, 1986a; Fitzgibons et al, 1987; Vasen et al, 1989; Houlston et al, 1990; Vasen et al, 1993). Rationale for this is based on evidence that screening for CRC in families with HNPCC has been shown to reduce the incidence of CRC, and decrease CRC deaths and overall mortality (Jarvinen et al, 1995; Vasen et al, 1998; Jarvinen et al, 2000). In a controlled 15-year trial, Jarvinen et al demonstrated a 65% decrease in the overall death rate in screened subjects compared to control subjects, all of whom were members of 22 HNPCC families, and at 50% risk of having a mutation. Planning screening for HNPCC is difficult because of the variable expression of the syndrome and non-penetrance of the gene. If no mutation is identified within a family, then current screening recommendations for at risk family members are based on clinical observations. This means that screening for extracolonic cancers may be included beginning usually 5- 10 years before the earliest cancer appears in the family (J. Green, personal communication).

1.241 Genetic Identification

Today we know that HNPCC is a genetically heterogeneous condition which can be identified presymptomatically by mutation testing (Peltomaki and de la Chapelle, 1997). Yet this is only possible for individuals in families that harbour an identified mutation in the mismatch repair (MMR) genes and unfortunately finding these mutations are difficult.

Microsatellite instability (MSI) (Originally referred to as replication error (RER), is defined as "a change of any length due to either insertion or deletion of repeating units, in a microsatellite within a tumour when compared to normal tissue" (Boland et al 1998). This characteristic was first observed in human CRC (Peinado et al, 1992; Ionov et al, 1993; Thibodeau et al, 1993). Previously bacteria and yeast studies identified new genes, named mismatch repair (MMR) genes, believed to play a vital role in DNA repair (Levinson and Gutman, 1987; Strand et al, 1993). MSI was noted in these organisms with mutations in the MMR genes. Therefore MSI was believed to result from defects in the MMR genes and human homologs of the yeast and Ecoli genes were then suspected to be involved in tumour formation in HNPCC (Strand et al, 1993).

Experimental evidence confirmed that mutations in MMR genes induce the mutator phenotype MSI (Loeb et al, 1994). MSI was reversed and MMR activity was restored when the normal copy of a MMR gene was reinserted into human cancer cell lines with deficient MMR (Koi et al, 1994). Biochemical investigations also supported these findings (Parsons et al, 1993; Umar et al, 1994).

Seven MMR genes including human mutS homolog 2 (hMSH2), human mutL homolog 1 (hMLH1), human mutS homolog 6 (hMSH6), human post-meiotic segregation 1 (hPMS1), human post-meiotic segregation 2 (hPMS2), human mutS homolog 3 (hMSH3), and human mutL homolog 3 (hMLH3) have been identified thus far. Linkage analysis of some large HNPCC families mapped the relevant gene to chromosome 2p16 (Peltomaki et al, 1993). The first human homolog was then cloned by two groups simultaneously and localised to 2p21-22 (Fishel et al, 1993; Leach et al, 1993) and it was hypothesized to be responsible for HNPCC (Strand et al, 1993). This hypothesis was confirmed by Leach et al, 1993 following the identification of hMSH2 mutations in HNPCC kindreds. Soon after a second HNPCC locus was mapped to chromosome 3p21 (Lindblom et al, 1993), and hMLH1 was cloned and localised to 3p21 (Bronner et al, 1994; Papadopoulous et al, 1994). Later, hPMS1 and hPMS2 were identified and localised to chromosome 2q31-33 and 7p22 respectively (Papadopoulos et al 1994; Nicolaides et al, 1994). The identification of hMSH6 (initially called the GTP binding protein) was reported in 1995 and it was mapped to chromosome 2p15 (Drummond 1995; Palombo 1995; Papadopoulos, 1995).

The hMSH2, hMLH1, hPMS2, and hMSH6 functions are required for effective MMR (Fischel, 1995). These genes encode proteins that function together with other proteins to repair mismatched bases at the S phase of the cell cycle (Kolodner et al, 1995). Each protein product plays a specific role in repairing damaged DNA. The proteins form heterodimeric complexes: hMSH2 with either hMSH6 or hMSH3 forms the mutS homolog alpha (hMutSα) complex; and hMLH1 either with hPMS2, hPMS1, or hMLH3 forms the mutL homolog alpha (hMutLα) to carry out the specific repair of the DNA.

The mutS homologs (hMSH2, hMSH6, hPMS2) recognise mistakes in the DNA (Palombo et al, 1996); hMSH2-hMSH6 binds to single-base mismatches, whereas hMSH2 in combination with another gene is postulated to bind extrahelical mismatched nucleotides (Acharya et al 1996; Alani et al 1996; Marsischky et al 1996). Following the recognition of the mismatches the hMutLα complex then joins the hMutSα complex to form the MutSα/MutLα complex. hMutLα assembles other proteins (involved in DNA synthesis) that complete the MMR system and the DNA is then repaired by removal and replacement of nucleotide sequences (Guerrett et al, 1999). It is not yet clear whether or how the loss of function of a gene affects the other genes in the heterodimers. Preliminary findings suggest that the hMSH6 protein is unstable in the absence of hMSH2 (Marra et al, 1998; Chang et al, 2000) and hMSH6 mutation carriers often show reduced levels of hMSH2 (de Wind et al 1999; Leeuw et al, 2000).

Pathogenic mutations in six of the MMR genes have been identified to be causative in cancer susceptibility (Papadopoulos et al 1994; Rhyu et al, 1996; Miyaki et al, 1997; Peltomaki et al, 1997; Wu et al, 2001). To date no mutations have been identified in hMSH3 (Fischel et al 1993; Lindblom et al 1993; Papadopoulous et al 1994; Huang et al, 2001; Lipkin et al, 2001). Currently more than 300 different mutations are identified in 500 kindreds worldwide (Peltomaki et al, 2001; International Collaborative Group on HNPCC, 2003). These include inactivating truncating mutations due to splice-site defects, frameshifts involving entire exons, nonsense mutations causing stop codons, and numerous missense mutations (Han et al, 1995; Kolodnel et al, 1995; Liu et al, 1995a; Luce et al, 1995; Nystrom-Lahti et al, 1995; Tannengard et al, 1995). The majority (90%) of mutations in HNPCC are attributed to the hMLH1 and hMSH2 genes

(Kinzler et al 1996, Liu et al 1996; Lindor et al, 1998). Mutations in MSH6 are suspected to be responsible for 5-10% of HNPCC (Kolodner et al, 1999; Wijnen et al, 1999) and mutations in PMS1 and PMS2 are expected to account for less than 5% of HNPCC (Liu et al, 1996).

Inheriting a mutation in one of the MMR genes does not guarantee the development of cancer. Instead this inheritance deems that an individual has a greater susceptibility to a cancer outcome. DNA MMR activity is sufficient with one functioning allele, i.e. a heterozygous state (Parsons et al, 1993). A cancer develops when the second allele (wild type) is also lost due to a somatic alteration (mutation or hypermethylation) (Bellamy et al 1995). The two hit hypothesis, originally purposed by Knudson, and applied to tumour suppressor genes, illustrates how inherited and somatic mutations collaborate in tumour formation (Knudson, 1971). With a normal MMR gene it would take two somatic mutations, one in each allele, for tumour development, whereas inheriting one defect at birth greatly increases the odds of developing a cancer because only one somatic mutation is needed. In vitro studies further supported this hypothesis when it was demonstrated that CRC cell lines with mutations in both alleles in hMLH1 (Papadopoulous et al, 1994; Parsons et al, 1995) or in hMSH2 (Umar et al, 1994), are deficient in MMR activity.

The pathway to developing a CRC in HNPCC appears to be different than in sporadic CRC (Liu et al, 1995a; Konishi et al, 1996). In HNPCC the adenoma to adenocarcinoma sequence is accelerated. An increased mutation rate is observed in MMR deficient cells compared to normal cells: the second hit induces the mutator phenotype (MSI) in the cell leading to the rapid accumulation of mutations in tumour

suppressor genes and oncogenes, and ultimately decreasing the time for formation of the adenocarcinoma (Ahlquist et al, 1995; Kinzler and Vogelstein 1996; Lynch et al 1996b). Potential differences are accounted for by observations of the different order and number of mutations occurring. For example, mutations in the APC gene (responsible for tumour initiation in sporadic cancer) have been shown to occur at a higher frequency in non-HNPCC tumours than in HNPCC tumours (Konishi et al, 1996, Salahshor et al, 1999), and mutations in the transforming growth factor β II receptor (TGFB-R11) , which occur in more than 90% of MSI tumours, probably play a role in tumour progression in HNPCC tumours and not in non-HNPCC tumours (Markowitz et al, 1995).

Also, somatic, rather than germline mutations in MMR genes may be responsible for the sporadic cancers with MSI (Liu et al, 1995a).

1.242 Clinical Identification

As a consequence to the genetically heterogeneous nature of HNPCC it is also a clinically heterogeneous condition. The identification of large HNPCC kindreds contributed much of what is now known about HNPCC. Analysis of these kindreds has demonstrated intrafamilial and interfamilial variables such as; ages of onset, sites of tumours, severity, and order of occurrence of HNPCC cancers (Green, thesis 1995). Beside CRC, numerous extracolonic malignancies are associated with HNPCC. Extracolonic cancers reported are varied and include malignancies of the endometrium, ovary, stomach, renal pelvis/ureter, upper biliary tract, skin (sebaceous tumours), small bowel, and brain (Watson et al, 1993; Aarnio et al, 1995; Vasen et al, 1996a; Vasen et al, 1999). Lynch et al (1993) showed increased frequency of endometrial, gastric, ovarian,

small intestine, biliary, uroepithelial, and kidney cancers. These extracolonic cancers appear in some but not all HNPCC families.

Variable expression of disease reported within and between families, in separate studies, may be due to geographical variation, different mutations, or modifier genes, but also could be affected by differences in methodologies such as different ascertainment methods. Overestimation and underestimation of cancer risk is possible because of selection biases. The subjects chosen may not be a true representation of the population. To further support the clinical spectrum of this syndrome, MSI and loss of MMR function has been identified in extracolonic tumours (Han et al, 1993; Risinger et al, 1993; Kobayashi et al, 1995).

1.243 Tumour Spectrum

CRC is the main manifestation of the syndrome, and diagnosis of HNPCC has typically occurred from identifying clusters of CRC cases (Lynch et al, 1988; Mecklin et al, 1991). Aarnio et al (1995) studying 293 putative mutation carriers in 40 AC families demonstrated that CRC was the most common tumour in HNPCC, including 190 of 293 (65%) of all tumours in their study. They reported a 78% lifetime risk of CRC in these families. A similar result was seen in a study by Green at al, (2002) in a study of 151 hMSH2 mutation carriers. By age 60, 77% of males had developed a CRC. Gender differences appear to occur as the risk of CRC was shown to be 2.8 times higher in males compared to females in a recent study of known mutation carriers (Green et al, 2002). The lifetime risk of CRC is similar in hMSH2 and hMLH1 mutation carriers (Vasen et al,

1994; Vasen et al, 1996a; Lin et al, 1998a) but may be lower in hMSH6 mutation carriers (Miyaki et al, 1997; Wijnen et al, 1999; Wu et al, 1999; Wagner et al, 2001).

Endometrial cancer is the most common extracolonic cancer in HNPCC (Mecklin et al, 1991). It is estimated that 5% of all endometrial cancer is caused by HNPCC (Vasen et al, 1990; Hakala et al 1991; Watson et al, 1993; Boyd et al, 1996). However, HNPCC families have been described that have either low frequency of endometrial cancers (Lynch and de la Chapelle, 1999) or none (Lynch et al, 1977; Lynch et al, 1988). The risk for endometrial cancer was shown to exceed the risk for CRC in female mutation carriers in some studies (Aarnio et al, 1995; Dunlop et al, 1997; Aarnio et al, 1999; Green et al, 2002). Aarnio et al (1995) demonstrated that the lifetime risk of endometrial cancer was 43% in female mutation carriers. Green et al (2002) reported a risk of 59% by age 60 in female carriers with a specific hMSH2 mutation. Such an observation may be gene/mutation specific. Lifetime risks of endometrial cancer were calculated to be 61% for hMSH2 mutation carriers and 42% for hMLH1 carriers (Vasen et al, 1996). Cumulative incidences of 36% in hMSH2 and 19% in hMLH1 of endometrial cancer were observed in a study of 105 known and putative mutation carriers (Lin et al, 1998). Although lower than reported for CRC, de Leeuw et al (2000), found MSI in 75% of HNPCC endometrial tumours.

Over the past century a decreasing incidence of stomach cancer has been observed in the western world. Environmental factors are believed to be responsible for this trend which may also be implicated in the increase of other types of cancer (Howson et al, 1986). The first description of HNPCC (originally referred to as Cancer Family Syndrome) reported more stomach cancer than CRC (Lynch et al, 1999). Watson et al

(1993) observed significant excess rates of stomach cancer in HNPCC compared to the general population. In Aarnio's study (1995), gene carriers were estimated to have a 19% lifetime risk of developing stomach cancer. Vasen et al (1996) studied hMSH2 and hMLH1 mutation carriers and reported an increased relative risk of 19.3 for stomach cancer in hMSH2 mutation carriers.

The hepatobiliary tract encompasses the liver and gallbladder. Watson et al (1993) reported significant excess rates of hepatobiliary tract cancer in HNPCC compared to the general population. Aarnio et al (1995) reported that gene carriers have an 18% lifetime risk of developing biliary tract cancer.

Numerous studies support an increase in transitional cell cancer of the renal pelvis and ureter in HNPCC families (Watson et al, 1993; Vasen et al, 1996a; Sijmons et al, 1998). Risk of bladder cancer does not appear to be increased in HNPCC (Watson et al, 1993; Sijmons et al, 1998; Vasen et al, 1996a). Watson et al (1993) reported significant excess rates of transitional cell cancers in HNPCC compared to the general population. Aarnio et al (1995) found that gene carriers have a 10% lifetime risk of developing urinary tract cancer. In Vasen's study (1996a) of mutation carriers, an extremely high relative risk of 75.3 was reported for renal pelvis/ureter cancers in hMSH2 mutation carriers.

Initially, Watson et al (1993) reported significant excess rates of ovarian cancer in HNPCC. This was followed by Aarnio's study (1995) on putative gene carriers where a 9% increased lifetime risk of ovarian cancer was demonstrated. However it has been noted that MSI rarely occurs in ovarian tumours (Fujita et al, 1995). In a recent review, ovarian tumours in HNPCC were compared to those in the general population and several

important findings were made. In this group, ovarian tumours occurred at an earlier age, were more likely to be epithelial in nature, and individuals were more likely to have a synchronous endometrial cancer (Watson et al, 2001).

Cancer of the small bowel is rare in the general population. Watson et al (1993) reported significant excess rates of small bowel cancer in HNPCC compared to the general population. A 1% (Aarnio et al, 1995) and 4% (Vasen et al, 1996a) increased lifetime risk of developing small bowel cancer has been reported in gene carriers.

The majority of pancreatic cancer is believed to be sporadic. However, the occurrence of pancreatic cancer in HNPCC has been described (Lynch et al 1985a; Vasen et al, 1990; Mecklin et al, 1991; Aarnio et al 1995; Dunlop et al ,1997) and without significant increased risk in HNPCC (Ponz de Leon et al, 1989; Watson et al, 1993). It is also associated with other cancer susceptibility syndromes where germline mutations in p16, BRCA1, BRCA2, and APC genes have been identified (Flanders and Foulkes, 1996).

No excess risk of brain cancer was reported in high risk members of 23 HNPCC kindreds (Watson et al, 1993). In contrast, Aarnio et al (1995) demonstrated a 1% increased lifetime risk of developing brain cancer in putative gene carriers. In another study, the relative risk for brain tumours was 6 times the amount seen in the general population when 14 brain tumours were reported in 1,321 subjects from 50 HNPCC kindreds (Vasen et al, 1996b). The occurrence of brain tumours and CRC was recognised in Turcot's syndrome which is characterised by colorectal polyposis, malignancies of the central nervous system, and café au lait spots. Now understood to be a molecularly heterogeneous disorder, both mutations in APC and MMR genes are believed to be

causative. Mutations in APC are associated with colonic polyposis and medulloblastomas seen in Familial Adenomatous Polyposis (FAP), while mutations in hMLH1 and hPMS2 are associated with CRC's and glioblastomas seen in HNPCC (Hamilton et al, 1995).

Like brain cancer, the significance of skin cancer in HNPCC was recognised through a syndrome, the Muir-Torre Syndrome (MTS). MTS is defined by the rare occurring sebaceous gland tumours, including sebaceous cell carcinomas and keratocanthomas (Hall et al, 1994a; Hall et al, 1994b), along with internal malignancies including CRC and extracolonic cancers observed in HNPCC (Muir et al, 1967; Torre et al, 1968). An autosomal dominant mode of inheritance is also observed. This led Lynch et al (1985b) to suggest that both MTS and HNPCC share a common genetic basis. MTS has been identified in large HNPCC kindreds (Green et al, 1994; Paraf et al, 1995). Honchel et al (1994) linked MTS to MMR genes by demonstrating MSI in skin lesions. The association to MRR genes was confirmed when MTS was mapped to the hMSH2 locus 2p22-21 (Hall et al, 1994b; Kolodner et al, 1994) and further when a germ-line mutation in exon 12 of hMLH1 was identified in a MTS kindred (Bapat et al, 1996).

Like stomach cancer the incidence of lung cancer has changed over time. It is now the number one cause of death due to cancer in both sexes. It is a fairly common cancer in the general population and is dramatically increasing in females (National Cancer Institute of Canada, 2002). On the other hand, a decreased incidence of lung cancer is observed in HNPCC kindreds (Pal et al, 1998). Currently no evidence exists to suggest that HNPCC kindreds are exposed to fewer carcinogens than the general public (Mecklin et al, 1986b).

Familial breast cancer is responsible for 6-19% of breast cancer cases seen in the general population (Colditz et al, 1993; Slattery et al 1993). Although breast cancer is most common in females, it does occur in males (Boyd et al, 1999). Two breast cancer genes BRCA1 and BRCA2 are identified and mutations in these are estimated to increase the lifetime risk of breast cancer up to 85%. Ovarian cancer is a common malignancy seen in mutation carriers of the same genes and a slight increase in prostate cancer has been reported in male mutation carriers (Ford, 1994; Thompson et al, 2001). There is little evidence that breast cancer occurs as a tumour in HNPCC (Watson et al, 1993), however an increased risk has been reported (Itoh et al, 1990). Several studies have shown breast tumours with MSI (Aldaz et al, 1995; Risinger et al, 1996) and a loss of the MMR gene MLH1 (Risinger et al, 1996). Wooster et al (1994) found that MSI occurs in approximately 10-20% of sporadic breast tumours. An explanation for the low occurrence of breast cancer in HNPCC is that it could occur as a low penetrant tumour (Boyd et al, 1999).

Several studies have supported the observation of multiple CRC's occurring in individuals in HNPCC kindreds (Mecklin and Jarvinen, 1993; Wijnen et al, 1998b; Box et al, 1999; Wang et al, 1999). Shah et al (1993) suggested that double primary tumours infer the presence of genetic susceptibility. A study by Box et al (1999) reported a 40% incidence of metachronous and 18% incidence of synchronous CRC in HNPCC at 10 years. Their study compared groups considered high risk for HNPCC (Amsterdam Criteria, suggestive HNPCC, and MMR defect) with population statistics. The HR group had rates significantly higher than general population rates for synchronous CRC (10.7%) and metachronous CRC (24.2%). This evidence supports the recommendation that a total

abdominal colectomy is justified in suspected HNPCC once a CRC has developed. Possibly in the future this recommendation will be based on genotyping.

1.244 Pathology

There are conflicting results concerning the use of pathological studies for the diagnosis of HNPCC. This is because of findings that a subset of sporadic CRC tumours display the MSI phenotype (Ruschoff et al, 1997) and also have similar clinicopathological features of HNPCC tumours displaying MSI. MSI tumours are usually proximally located, poorly differentiated (Jass et al, 1994; Ruschoff et al, 1997), mucinous, with neuroendocrine differentiation (Lynch et al, 1991; Lynch et al, 1993), diploid (Kouri et al, 1990; Frei et al, 1992; Losi et al, 1995), and signet-ring-cell-type carcinomas (Kim et al, 1994).

It is suggested that a better survival is observed with certain types of pathology and this pathology is associated with HNPCC (Kokal et al, 1986; Schutte et al, 1987) or tumours displaying MSI (Lothe et al, 1993; Thibodeau et al, 1993; Berney et al, 2000). The nature of DNA content has been compared in HNPCC and sporadic CRC. The impression is that HNPCC tumours tend to be diploid in nature and sporadic CRC tends to be aneuploid. Diploid tumours behave less aggressively supporting a better survival (Losi et al, 1995). Also, a certain protective immune response (Branch et al, 1995) has been postulated to play a role in the observed favourable outcome.

As adenomas are premalignant lesions they are associated with an increased risk of CRC. It is difficult to accurately determine the prevalence of adenomas in HNPCC. Nonetheless, a general consensus seems to be that the presence of adenomas increases

one's risk of CRC. Pathologists could draw attention to an individual at increased risk which could lead to better surveillance and removal of future adenomas in their premalignant state.

1.245 Genotype-phenotype Correlations

Today it is possible to estimate genotype-phenotype correlations and researchers are using identified mutation carriers from large HNPCC kindreds to achieve this. These correlations are poorly understood due to the phenotypic variation observed in a growing number of identified mutations, and because no mutation has given a distinctive phenotype. Numerous studies have been completed from various populations. Preliminary evidence suggests that extracolonic cancers occur more frequently in hMSH2 than hMLH1 mutation kindreds (Vasen et al, 1996a; Jager et al, 1997; Lin et al, 1998; Peltomaki et al, 2001). A recent study by Green et al (2002) looked at the impact of gender and parent of origin on the phenotypic expression of HNPCC in a large Newfoundland kindred with a common hMSH2 mutation. The study concluded that the mutation was passed on by women more than by men; women were more likely to develop and die of CRC if they received the mutation from their fathers; and that men developed CRC and died of cancer earlier than women. It also estimated age of onset of certain cancers. Studies of this nature not only provide insights into the heterogeneous nature of HNPCC but also help improve the surveillance and care of known mutation positive HNPCC family members. However, many more questions arise demonstrating the complexity of the research. For example a variable age of onset has been noted in family members with the same mutation and also in different families with the same

mutation (Liu et al, 1996; Peltomaki et al, 2001); one quarter of extracolonic cancers in the mutation carriers were non-HNPCC tumours (Peltomaki et al, 2001).

Of particular interest is the finding that a mutation in the hMSH6 gene leads to features atypical of HNPCC. This is characterised by a reduction in CRC, an increase in endometrial tumours and a delayed age of onset of both (Wagner et al, 2001). Wagner et al (2001) reported an older mean age of onset than previously reported for HNPCC, age 55 for CRC and age 55 for endometrial cancer. Other studies have also reported the delayed age of onset for hMSH6 by comparing hMSH6, hMSH2, and hMLH1 mutation carriers with CRC (Vasen et al, 1996) and with endometrial cancer (Wijnen et al, 1999); the mean age of CRC was 50 years for hMSH6 compared to 44 (hMSH2) and 41 (hMLH1), and the mean age of endometrial cancer was 53 years (hMSH6) compared to 49 (hMSH2) and 48 (hMLH1). Also the presence of MSI is observed less frequently in hMSH6 mutation carriers; tumours are either completely stable or display another mutator phenotype, a low MSI (Wu et al, 1999).

1.246 Penetrance

Penetrance is the probability that a gene will have any phenotypic expression at all. A reduced penetrance means that not all gene carriers will develop a cancer. Aarnio et al (1995) estimated that more than 20% of mutation carriers will never develop a CRC. Several studies have attempted to address the issue of penetrance (Aarnio et al, 1995; Vasen et al, 1996a; Vasen et al, 1996b; Dunlop et al, 1997; Lin et al, 1998b; Froggatt et al, 1999; Green et al, 2002), yet the level of penetrance remains unknown, although one suggestion is that there is a 92% penetrance by age 75 and 57% by age 50 (Vasen et al,

1996a). To add to the difficulty, inter gene variation is reported. Wagner et al (2001) reported on a large hMSH6 kindred that showed a reduced penetrance of CRC; only 7% and 32% of those affected had developed a CRC by age 50 and age 80 respectively. Both genetic and environmental modifying factors may a play a role in reduced penetrance. This has implications for the recommendation of preventative surgery for known or putative mutation carriers and leads to complications in genetic counselling.

1.3 Other Hereditary Syndromes at Increased Risk for CRC

Unlike HNPCC, Familial Adenomatous Polyposis (FAP) usually has a distinguishable phenotype. It is characterised by the presence of hundreds to thousands of polyps. In contrast to HNPCC, a higher rate of adenoma formation, but a normal adenoma to carcinoma progression, is observed in FAP (Vogelstein and Kinzler, 2002). FAP is not a cancer diagnosis but can lead to an increased cancer risk for an individual. The risk is believed to be extremely high as a 100% penetrance is believed for mutation carriers. FAP is caused by germline mutations in a tumour suppressor gene, the APC gene (Groden et al 1991; Nishisho et al, 1991). These mutations usually cause truncation of the encoded protein that can result in many different phenotypes; extracolonic manifestations of the disease include retinal lesions, osteomas, epidermal cysts, desmoid tumours, and brain tumours. Also, an attenuated form of FAP (AFAP) exists where only a small number or more variable number of polyps develops (Spirio et al, 1993; Lynch et al, 1995).

Investigations of the APC gene and FAP have lead to an improved understanding concerning mutations in the APC gene which are believed to be necessary for tumour

initiation in both FAP and sporadic cancer (Kinzler and Vogelstein, 1996). Studies of FAP have attributed to Knudson's "two-hit" hypothesis that explains the necessity of the loss of the wild type allele by somatic mutation enabling tumour initiation. Those identified as carriers of an APC mutation or manifesting the phenotype (colonic polyposis) should undergo prophylactic colectomy as a preventative measure. Genetic counselling and genetic testing is recommended for at risk individuals. Flexible sigmoidoscopy or colonoscopy for AFAP families is recommended at puberty for mutation positive individuals, or for those at risk in families with unknown mutations. In Newfoundland, at least 25 families are identified with classical FAP or AFAP. Five families are known to harbour an AFAP "founder" mutation and 2 families have 2 of the most common (20% of total known) FAP identified mutations. To date the other families have no mutation identified (J. Green, personal communication).

Peutz-Jeghers syndrome (PJS) is characterised by multiple gastrointestinal hamartomatous polyps of the gastrointestinal tract, melanocytic macules, an early age of onset and autosomal dominant transmission (Spigelman et al, 1989). Pathology should be able to identify the specific polyps that usually form in the jejunum and lead to intussusception of the small bowel (Tomlinson et al, 1996). Although this blockage usually occurs before a cancer develops (Tomlinson et al, 1996), the affected individual or other family members have a high risk of developing various types of adult onset cancer including breast (54%), colon (39%), pancreas (36%), stomach (29%), and ovarian cancer (21%) (Giardiello et al, 2000). Germline mutations in a suspected tumour suppressor gene (STK11) have been identified in approximately half of recognised PJS families (Hemminiki et al, 1998; Jenne et al, 1998). The variable phenotype has led to

the difficulty of identifying this rare syndrome. In Newfoundland one family is known to the Medical Genetics program but PJS probably occurs in as many as 5 families (J. Green. personal communication).

Juvenile polyposis syndrome (JPS) is an extremely rare condition that presents in childhood and that was originally believed to occur sporadically (Bussey et al, 1978). It is characterised by gastrointestinal hamartomatous polyps. Mutations in the germline of the MADH4 gene and in the gene-encoding bone morphogenic protein receptor 1A (BMPR1A) are responsible for the autosomal dominantly pattern that is observed in some cases (Friedl et al, 2002). An increased risk of CRC has been reported in patients with JPS (O'Riordain et al, 1991). Its occurrence in the Newfoundland population is not reported (J. Critch, personal communication; J. Green, personal communication).

1.26 Variation in Identifying HNPCC

A fast developing molecular basis for the identification of HNPCC has led to a wide variation of opinion in the literature as to how best to identify HNPCC. Methods currently used to identify HNPCC include the use of a variety of clinical and molecular techniques.

1.261 Clinical Criteria

The first criteria used to identify HNPCC (originally referred to as Cancer Family Syndrome) were proposed by Henry Lynch (Lynch et al, 1981). The criteria were based on decades of research where large families were identified who had manifested the occurrence of cancers over generations. The criteria were general and non-specific,

including early age of onset, proximal location of colonic tumours, multiple primary tumours, vertical genetic transmission, common occurrence of endometrial and other adenocarcinomas in a kindred.

International clinical criteria for the diagnosis of HNPCC have since been established (Vasen et al, 1991; Rodriguez- Bigas et al, 1997). This was first developed in 1990 by an international group of researchers know as the International Collaborative Group for HNPCC. The criteria were called the Amsterdam Criteria I (AC I):

i. at least three relatives with histologically verified colorectal cancer, one of them being a first degree relative of the other two (FAP excluded); and
ii. at least two successive generations affected; and
iii. in one of the individuals, diagnosis of colorectal cancer before age 50

The criteria would enable researchers from different geographic areas to present their HNPCC patients and would be instrumental in the identification of mutations.

The establishment of these criteria was initially accepted but since that time it is widely criticized. The criterion does not consider extracolonic cancers, family size, and reduced penetrance (Percesepe et al, 1994, 1995; Ponz de Leon, 1996; Beck et al 1997; Bapat et al 1999). The introduction of these strict criteria into clinical practice may result in failure to identify true HNPCC families. Individuals and their family members would miss valuable genetic counselling and screening. The identification of many mutations would be missed. However, the criteria were originally designed to eliminate those who did not have the condition (specificity) rather than to identify those who did (sensitivity) (Vasen et al, 1999), to guide the search for relevant genes, not to guide clinical practice.

Researchers continue to develop their own sets of minimum criteria for their publications. Many similarities exist between the criteria. Examples include the Modified Amsterdam Criteria (Bellacosa et al, 1996) which includes:

i. two CRC cases in first-degree in very small families that cannot be explained further;
ii. CRC affecting more than one generation; at least one CRC case diagnosed before age 55 years

OR

i. two first degree relatives affected by CRC, plus
ii. a third relative with an unusually early-onset neoplasm or endometrial cancer.

Japanese criteria established the following criteria (Fujita et al, 1996):

i. three or more CRC cases among first degree relatives;
ii. two or more CRC among first-degree relatives and any of the following: diagnosis before age 50 years; right colon involvement; synchronous or metachronous multiple CRC; and association with extracolonic malignancy.

Other groups have used the AC 1 criteria and their own less strict criteria to capture families that might be suspected of an inherited condition. For example the Korean Hereditary CRC Registry used the AC 1 and their own "suspected HNPCC" criteria which includes:

i. vertical transmission of CRC or at least two siblings affected with CRC in a family;
ii. development of multiple CRC tumours or at least one CRC diagnosed before the age of 50 years (Han et al 1996).

In order to compensate for the narrow criteria, the ICG-HNPCC, in 1998, revised the existing criteria and developed a new set of criteria that includes extracolonic cancers (Vasen et al 1999); this is known as the "The Amsterdam Criteria II" (AC II).

This includes:

i. at least 3 relatives with an HNPCC-associated cancer (CRC, cancer of the endometrium, small bowel, ureter, or renal pelvis);
ii. one should be a first-degree relative of the other;
iii. at least 2 successive generations should be affected;
iv. at least 1 should be diagnosed before age 50 ;
v. FAP should be excluded in the CRC case(s) if any ;
vi. tumours should be verified by pathological examination

The HNPCC cancers included in this criterion were CRC, endometrial, small bowel, ureter, or renal pelvis. These cancers were selected, after a review of the literature (Watson et al, 1993; Aarnio et al, 1995; Vasen et al, 1996a; Vasen et al, 1996b; Sijmons et al, 1998; Vasen et al, 1999), because they demonstrated the highest relative and lifetimes risks in HNPCC kindreds. Other cancers, stomach, ovary, brain, and hepatobiliary tract were noted to be associated with HNPCC (Watson et al, 1993; Aarnio et al, 1995; Vasen et al, 1996a; Vasen et al, 1996b; Sijmons et al, 1998; Vasen et al 1999). There was some debate over exclusion of these other extracolonic cancers. For example, stomach cancer presented more often in Asian compared to western HNPCC kindreds. The ICG-group decided that establishment of these criteria, and any future changes, should not be country specific. The criteria also omitted the need to identify CRC in kindreds. This was based on the presentation of kindreds with endometrial cancer which were also found to have MMR mutations (Wagner et al, 2001).

1.262 Molecular Identification

Tumour microsatellite instability (MSI) analysis is currently the primary approach used for the selection of CRC patients who that should undergo mutation analysis (Liu et al 1996; Aaltonen et al, 1998). As referred to earlier, MSI analysis may serve as a

prognostic marker. Also a recent study has demonstrated different responses of chemotherapy in MSI-high (MSI-H) and MSS tumours (Elsaleh et al, 2000). Microsatellite instability analysis utilises PCR for the detection of microsatellites that are present in tumour DNA and absent in normal DNA. MSI-H is reported in 63 to 92% of HNPCC tumours (Jass et al, 1995; Liu et al, 1996; Calistri et al, 2000; de Leeuw et al, 2000) compared to reports of 12 to 28% of sporadic tumours (Aaltonen et al 1993; Ionov et al 1993; Lothe et al 1993; Thibodeau et al, 1993; Young et al 1993; Aaltonen et al 1994b; Kim et al 1994; Calistri et al, 2000). Therefore MSI analysis lacks specificity for HNPCC (Aaltonen et al, 1994b). Cunningham et al (2001) suggested that the pathway leading to carcinogenesis in sporadic MSI-H CRC is probably different than in HNPCC CRC. Instead of germline defects in MMR genes an epigenetic mechanism causing hMLH1 inactivation may account for the MSI-H findings in sporadic CRC (Kane et al 1997; Cunningham et al 1998; Herman et al 1998; Veigl et al, 1998; Wheeler et al 1999). This inactivation is caused by promoter hypermethylation of both hMLH1 alleles at the somatic level and probably accounts for approximately 8-12% of all CRC (Cunningham et al 2001).

Although the majority of tumours in HNPCC families are MSI-H, tumours have been found in HNPCC families that are microsatellite stable (MSS) (Farrington et al, 1998; Bapat et al, 1999; Debniak et al, 2000; Liu et al, 2000). The presence of MSI-H is reported in CRC and extracolonic cancers (Risinger et al, 1993; Aaltonen et al, 1994).

The Bethesda guidelines were established to identify individuals who may benefit from further investigation, the first of which would be MSI analysis (Rodriguez-Bigas et al, 1997). Individuals must meet one of seven criteria.

These include:

i. individuals with cancer in families that meet the Amsterdam Criteria;
ii. individuals with two HNPCC-related cancers, including synchronous and metachronous CRC or associated extracolonic cancers;
iii. individuals with CRC, plus CRC and/or HNPCC related extracolonic cancer and or/colorectal adenoma in a first-degree relative; at least one of the cancers diagnosed before age 45 years and the adenoma diagnosed before age 40 years;
iv. individuals with CRC or endometrial cancer diagnosed before age 45;
v. individuals with right-sided colorectal cancer with an undifferentiated histopathological pattern (solid/cribiform) diagnosed before age 45 years;
vi. individuals with signet-ring cell type CRC diagnosed before age 45 years;
vii. individuals with colorectal adenomas diagnosed before age 40 years.

Confusions in the literature led the National Cancer Institute in 1997 to sponsor "The International Workshop on Microsatellite instability and RER Phenotypes in Cancer Detection and Familial Predisposition." The goals of this workshop were to review the current knowledge and to set guidelines to direct researchers so that comparison of data is possible at an international level. Five microsatellite markers were recommended and definitions for microsatellite analysis were agreed upon: CRC tumours are microsatellite stable (MSS) if no markers show instability, microsatellite instable low (MSI-L) if 1 of 5 markers show instability, and microsatellite high (MSI-H) if 2 or more show instability (MSI-H >30% of loci examined, MSI-L <30% of loci examined, MSS no instability at any site (Boland et al 1998).

An abundance of information in a short period has resulted in difficulties in determining the diagnostic utility of the previously mentioned criteria. Syngal et al (2000) reported on the sensitivities and specificities of the AC I, AC II, modified AC, and the Bethesda Criteria for the detection of MMR mutations. The sensitivity of the AC I was 81% (95% CI 43-79) and specificity was 67% (50-85). Sensitivities of the modified

AC I and AC II were 72% (95% CI 58-86) and 78% (95% CI 64-92) respectively. The sensitivity of the Bethesda Criteria was 94% (95% CI 88-100) and the specificity was 25% (95% CI 14-36). The authors increased the specificity of the Bethesda guidelines to 49% (95% CI 34-64) when they only included the 1st three criteria. They concluded that the latter approach was the most sensitive for the detection of MMR mutations. However the value of these criteria has not been established in a population based study. MSI-H is a sensitive predictor for the presence of MMR mutations but not specific to HNPCC as 15% of sporadic CRC's also show MSI-H (Aaltonen et al, 1994b).

Recently a new technique has been used in laboratories studying HNPCC. Immunohistochemistry (IHC) analysis is a technique which determines if a MMR protein is present or absent in tumour tissue by staining for antibodies which recognise the proteins. Lindor et al (2002) demonstrated that IHC analysis is specific (100%) and also highly sensitive (92.3%) for the detection of MMR mutations (all IHC-deficient tumours were MSI-H whereas MSS tumours did not show loss of protein expression). This study only tested tumours for deficiency of hMLH1 and hMSH2. Also, tumour samples were selected from HNPCC registries which biases the analysis (therefore cannot be applied to the general population).

Correlation between MSI and IHC analysis has been reported to range from 75% to 100% (Lindor et al, 2002). Fujiwara et al (1998) stained for hMSH2 in 39 selected MSI-H tumours from twenty HNPCC families (12 families were known mutation carriers) and reported sensitivity for germ-line mutations of hMSH2 of 86%, specificity of 93%, a positive predictive value of 92%, a negative predictive value of 88%, and overall accuracy of 90%.

Mutation detection can be done by several different methods, none of which are 100% sensitive (Eng et al, 1997). The apparent choice for mutation detection in HNPCC has been exon-by-exon genomic sequencing, though this technique probably misses about 20% of all mutations. Southern hybridization has been used to detect large deletions (Wijnen et al 1998a) and a new technique, MLPA (multiplex ligation-dependent probe amplification), has recently been developed for the detection of genomic deletions detection (Gille et al, 2002). Inter gene variation is observed in the size of the gene and the types of mutations seen. For example, large deletions are common in hMSH2 and missense mutations are common in hMLH1.

A definitive diagnosis of HNPCC can only be established by demonstrating a germline mutation (Peltomaki et al 1997). In approximately half of the families that meet the clinical (Amsterdam) criteria, germline HNPCC gene mutations have not yet been identified (Liu et al, 1996; Weber et al, 1997; Sijmons et al, 1998). However, germline mutations have been found in families not fulfilling the AC (Moslein et al, 1996; Nystrom-Lahti et al, 1996; Wijnen et al, 1997; Genuardi et al, 1998) and also in patients with early onset CRC and no family history (Liu et al, 1995b).

1.27 Population-Based Registries

Registries are an invaluable source for the collection of vast amounts of data. A registry's aims include improving screening, early detection and treatment, and education about the particular condition. The registry will uphold these aims by collecting and updating relevant data and adapting these methods as science and technology improves. The use of registries enhances epidemiological research both retrospectively and

prospectively and does this in an interdisciplinary manner. The collaboration of numerous registries is possible when there is a need to increase the population size and to compare trends in populations.

Registries have been used extensively for the management of hereditary syndromes (Jarvinen et al, 1984; Vasen et al, 1990) which has resulted in improved outcomes. For example, a decrease of mortality due to cancer in FAP from 57% to 6%, over 14 years is attributed to the development of the Ontario FAP registry (Madlensky et al, 1995). Population-based CRC and HNPCC registries exist in numerous countries such as Australia, Canada, Denmark, Finland, Italy, Japan, and United States. These registries are able to document and expand the family histories of incident CRC cases. By expanding family histories they will determine the contribution of hereditary cancers to the total cancer burden (Ponz de Leon, 1993) which in turn would lead to better services. The data collected includes a range from demographic information, family history, medical records, and now mutation testing results. Certain governmental regulations may exist to protect individual's right to privacy. Usually registrants in these specialised registries sign consent forms to be registered, and consent to the release of medical and family information, DNA and pathological specimens.

1.28 Population-Based Studies

Specialised population based HNPCC studies began in the late 1980's. The goals of these studies were to determine the frequency of HNPCC in the general population. Population-based studies provide an unbiased method of case ascertainment thus leading to improved estimates of HNPCC both at a clinical and molecular level. The variety of

methods utilised in these studies cover a variety of definitions of HNPCC and definitions of a population. Primarily, a variety of criteria has been used to collect and interpret family history data.

An early study of the Finnish population, using 3 cases of CRC in first degree relatives as a clinical definition of HNPCC , reported that HNPCC was responsible for 3.8-5.5% of all CRC and 29.4% of all CRC less than 50 years old (Mecklin et al, 1987). One of the first population based studies using the Amsterdam Criteria I reported on the experiences of an Italian registry after 6 years in existence. Using the AC I they reported a frequency of HNPCC 3.4 -4.5% (Ponz de Leon et al, 1993). A retrospective and prospective study conducted in the UK utilised the AC I, and less strict criteria which included 3 cases of HNPCC cancers at any age. Of 1137 consecutive cases, 0.3% (n=3) fulfilled the AC I, and 1.4% (n=16) fulfilled their less strict criteria (Evans et al, 1997).

Identifying HNPCC families has enhanced the ability to identify unique mutations in specific populations. Due to the recent advances in molecular genetics the methods to identify HNPCC have expanded to include a combination of microsatellite analysis, IHC analysis, and mutation testing. Salovaara et al (2000) reported on the population based molecular detection of HNPCC in Finland. Of 535 consecutive CRC cases tested by MSI analysis, 66 (12%) were MSI-H and 18 (3.4%) of the 66 had germline mutations in either MLH1 or MSH2. Only 3 of the 535 cases fulfilled the AC I, and another 2 cases fit more relaxed criteria. One of the 5 tumours had MSS and a germline mutation was not detected. The authors combined their data with a similar previous study (Aaltonen et al, 1998) in the Finnish population for a total of 1044 cases. They reported that germline mutations account for 2.7% (n=28) of CRC in their population.

Percesepe et al (2001) reported on the population-based molecular screening for HNPCC in an Italian region. Of 336 cases that were tested by MSI analysis, 28 (8.3%) were MSI-H. IHC analysis revealed lack of expression of the hMLH1 protein in 20 of the 28 MSI-H cases (71.4%). Promoter hypermethylation of the hMLH1 gene was implicated in 14 (70.0%) of these MSI-H cases. Although hMSH2 expression was normal in all cases, one (0.3%) case had a germ line mutation in this gene. The authors explained that the mutation occurred at the COOH terminus causing expression of the protein during IHC analysis. The most recent study (Katballe et al, 2002) reported an even lower HNPCC frequency of 1.1% in the Danish population. Subjects were classified as HNPCC if they were either AC1 or AC2 along with MSI- H or MSI-L and/or harboured an hMLH1 or hMSH2 mutation.

1.281 Canadian Studies

An early population-based study reported on the frequency of HNPCC in Southern Alberta. The selection criteria were all CRC's diagnosed in individuals less than age 50 over a 15 year period. They identified HNPCC in 12 families (3.1%). They also proposed that if less strict criteria for HNPCC were used then another 25 families could have been added to their total with a HNPCC frequency of 9.5% (Westlake et al 1991).

Ontario developed a CRC registry in 1997. It is the first population-based family CRC registry to be developed in Canada. Their specialised registry was created on the basis of the need to strengthen strategies for managing high risk CRC which in turn would strengthen the management of sporadic CRC. It mirrored existing breast cancer

registries within the province. The many components and goals of the registry include: determining the genetic epidemiology of CRC, gene discovery, primary prevention, psychosocial research, screening, and treatment. This would be accomplished by improving resources for CRC research in a multidisciplinary approach. Cotterchio et al (2000) reported on their first year experiences. They had a 61% response rate and found that low risk families were less likely to participate. A high participation rate is necessary in order to ensure that information obtained in the study is applicable to the general population. An approximately 65% response rate has been reported in other CRC studies (Le Marchard et al, 1999; Kampman et al, 1999). Issues preventing participation include time commitments, confidentiality, high mortality rates, and selection. A population-based study of this nature has not been carried out in Newfoundland.

Newfoundland is accredited with the reputation of an attractive place to conduct genetic research. Little out migration, after settlement of the province began in the 1700's, has resulted in Newfoundland's unique genetically isolated population (Bear et al, 1987). The cooperation of large families has provided the means to identify and define inherited conditions in Newfoundland. For example, a cluster of four large kindreds have been identified that have a Multiple Endocrine Neoplasia, type 1 (MEN-1) founder mutation, a common nonsense mutation (R460X) (Olufemi et al, 1998). Similar clinical manifestations of MEN-1 in the 4 families from a common geographic location led to the discovery of this founder mutation. Linking of these large families to a common ancestral location was possible from analysis of phenotypes (Green, thesis 1995). Ongoing research into other hereditary disorders, including polycystic kidney disease, von Hippel-

Lindau disease, and cardiomyopathies, are providing unique insights into the clinical and molecular management of these autosomal dominant hereditary conditions worldwide.

Newfoundland HNPCC families have been described by a number of key individuals within the province. Dr. Jane Green and Dr. Roger Green are two geneticists in Newfoundland who have had an interest in HNPCC. Currently there are 41 HNPCC families identified within the province. There are numerous others that are suspected HNPCC families. The identification and collection of family information has contributed to the development of improved services and to a better understanding of hereditary CRC within the province and also worldwide. The discovery of a large Newfoundland HNPCC kindred was crucial in mapping the first HNPCC gene, hMSH2 (Peltomaki et al, 1993; Leach et al, 1993). The mutation, an A-T transversion at the +3 position of the splice-donor site of exon 5 (referred to as the "family C" mutation in Newfoundland) is the most common recurring hMSH2 mutation worldwide and was shown to have a founder effect in Newfoundland. Following discovery of the same mutation in American and European families, haplotype analysis disproved the worldwide founder-effect (Froggatt et al, 1999). In Newfoundland the mutation occurs in 13 families, 4 of whom have been traced back to an original ancestor. Individuals in these families have and can have pre-symptomatic gene testing to identify those who are at greater risk of developing a cancer. Approximately 176 individuals are carriers of the "family C" mutation. Currently, screening for the "family C" mutation is recommended for all Newfoundland HNPCC families. This family has been used in analysis by researchers from around the world (Aaltonen et al, 1993; Froggart et al, 1999; Green et al, 2002).

1.3 Purpose

The purpose of the pilot study is to determine the proportion of hereditary CRC within the Avalon Peninsula, Newfoundland, over the period 1997-1998. In addition, the objective is to discover possible obstacles that will impede the development of a Newfoundland Familial Colorectal Cancer Registry.

Primary Research Objectives

1. To determine the proportion of Hereditary vs. Sporadic CRC cases on the Avalon Peninsula.
2. To identify families affected by inherited CRC.
3. To describe the molecular characteristics of Hereditary vs. Sporadic CRC.
4. To evaluate current methods of diagnosing HNPCC.

CHAPTER 2: METHODS

2.1 Design

This is a population-based study in which CRC probands were identified retrospectively during a given time period and two investigations were undertaken including a family history study and laboratory study of CRC probands, using DNA and tumour. Joint Masters Degree and PhD Degree projects in Clinical Epidemiology and Medical Genetics were designed in which the family history studies were performed by myself and the laboratory studies were performed by Angela Hyde. In this report, only the laboratory results in those with completed family histories will be reported. Only a brief description of the laboratory methods will be given here as they will be reported in detail in the PhD thesis, of Angela Hyde, to be prepared in the near future.

2.2 Ethical Considerations

Ethics approval was granted by the Human Investigation Committee (HIC) of the Faculty of Medicine, Memorial University of Newfoundland following a review of the proposal (Appendix B). Approval for the project was also obtained from the two Health Boards involved in the study: the Health Care Corporation of St. John's (Research Proposal Approval Committee) and the Avalon Peninsula Health Board (see Appendix B).

A number of concerns arose while applying for ethics approval. One of the objectives of this pilot project was to obtain ethics approval that would establish the process for ethics applications for similar projects in the future. The nature of the study

was instrumental in developing a template for future studies of its type in Newfoundland. The first application was submitted on July 24th, 2000 and full approval for the project was given on Oct 12, 2000.

The application included a detailed description of the objectives, rationale, data collection, time commitment, finances, study consent form, and study materials of the project. Included in the consent form was consent to participate in genetic research. The participants were informed of measures to be taken in order to ensure their privacy. There were no identifiable risks to participants except a blood sample taken may cause some bruising. Participants could withdraw from the study at any time and all family history data collected to that point would be destroyed however, all clinical and demographic data collected to that point would not be destroyed but kept in the database.

2.3 Family History Study

2.31 Study population

The population studied included all incident CRC cases in 1997 and 1998, identified through the provincial tumour registry at the Newfoundland Cancer Treatment and Research Foundation (NCTRF). A review of the meditech coding system (a regional hospital reporting tool), and of pathology reports was also used in order to identify other cases that may have been overlooked by the registry. The Newfoundland Center for Health Information has developed population estimates for each Health Board Region. Health Board regions 1 and 2 are found on the Avalon Peninsula, and include approximately half (250,000) of the Newfoundland and Labrador population. Because

half of the island's population resides on the Avalon Peninsula, and extensive travel to the rest of the province was not possible, these Health Board regions were chosen for this study. The Health Science Centre, St. Clare's Mercy Hospital, The Salvation Army Grace General Hospital, and Carbonear General Hospital served the region at the time. The Salvation Army Grace General Hospital closed in 2000. The most recent year with completed information in the NCTRF registry was 1998. We identified 179 potential probands for the years 1997 and 1998 who fit the inclusion and exclusion criteria.

2.311 Inclusion Criteria

Eligible cases were defined as all males and females diagnosed with CRC (ICD9 codes 153 and 154), in either 1997 or 1998 who were between the ages of 20-69 inclusive, and resided in either Health Board Region 1 or Health Board Region 2. If a proband was deceased an appropriate next of kin was asked to participate for the collection of the family history. Some probands had previously been identified as having a hereditary cancer by the Medical Genetics program here at Memorial University. These individuals and the data collected by the Medical Genetics group were included in the study.

2.312 Exclusion Criteria

Ineligible cases were defined as CRC cases with age greater than or equal to 70, and those who did not reside in either Health Board regions 1 or 2 at time of diagnosis.

2.313 Ascertainment of Study Participants

The NCTRF registry records all cases of cancer as well as diagnosis information, demographic data, treatment data, and physician data. All attending physicians and family physicians when necessary were contacted in order to obtain consent from individuals eligible for the study (See Appendix C). A proband was excluded if the physician making the initial contact felt that the individual was not appropriate for this study. In the case of a deceased proband, a next of kin was identified by the physician to serve as a proxy.

Initial contact with the patient or next of kin was made by the physician. A standardised letter (Appendix D) was developed to aid the physician in this process. This letter introduced the project and the research team. The letter also provided the potential participant with an opt in or opt out method of study enrolment. The individual did this by contacting the physician's office or the research team by telephone. Those who did not respond were called by a third party (an employee of the NCTRF) and asked if they were interested in participating in the study. After oral consent was obtained, a consent form was sent to the participant (Appendix E). The consent form explained the study in detail. After the signed consent form was returned, an appropriate time was arranged for an interview with the study participant.

2.314 Baseline Characteristics

The NCTRF registry and a review of medical charts were used for the collection of baseline characteristics which included demographic and clinical data. An extraction form (Appendix F), identifying the proband only by a unique identity number, was

constructed and used to collect this data. The baseline characteristics included: birth place, residence, sex, age, age of diagnosis, status (alive or dead), pathological descriptions of tumours, symptoms leading to diagnosis, named treating physicians, diagnosing and treating hospitals, and treatments.

The tumour was defined as occurring in the proximal colon if it was located in the cecum, ascending colon, or the transverse colon, and the tumour was defined as occurring in distal colon if it was located in the descending or sigmoid colon or in the rectum.

2.32 Collection of Family History Data

An interview was held at the convenience of the participant. Participants were interviewed at the Health Sciences Centre, Carbonear General Hospital, or the participant's residence. Some participants requested to fill out a form and others completed a telephone interview. During the interview details of the study were described and the participants were given the opportunity to ask questions or discuss any concerns. At this time they were also asked to read and sign the Newfoundland and Labrador Medical Genetics program consent form (Appendix D) and a release of medical information form (Appendix D). A standardised form for the collection of family histories was provided by the Medical Genetics program, and was used as a guide for the collection of a three generation pedigree. Details about types of cancer, age at diagnosis, date of birth, current age, age of death, cause of death, other related medical information, place of birth, and place of residence were recorded about family members. A brief medical history, reported by the proband, including symptoms leading to diagnosis, treatments and any other medical history was recorded.

At this time, the importance of reviewing the medical charts of possibly affected family members was explained to the study participant, in an attempt to include their family member's participation in the study. Participants were provided with copies of a release of medical information form to give to family members who were affected with a cancer. Participants previously referred to the Medical Genetics program were re-interviewed where possible. Permission was obtained to review the Medical Genetics chart and information obtained in an interview was added to that obtained from the chart.

2.33 Primary Outcome Family History Study

The primary outcome of interest was the risk of HNPCC in a proband and his/her family. A proband's three generation family history was collected to determine this risk: first degree relatives include siblings, offspring and parents; second degree relatives are aunts, uncles, nieces, nephews, grandparents and grandchildren; cousins are third degree relatives.

2.34 Classification of Primary Outcome

Pedigrees were constructed using the computer package Cyrillic 2.1. Under the supervision of Dr. Jane Green, probands were classified as having hereditary or sporadic cancer. This included 3 risk categories for HNPCC: 1) high risk; 2) intermediate risk (as defined by the OFCCR); 3) low risk. It was also noted if a proband potentially had FAP.

HNPCC Risk Criteria

HIGH RISK

- Amsterdam Criteria

  At least 3 family members with CRC; one is a first degree relative of the other two; two consecutive generations represented; at least one individual younger than 51 years at diagnosis; FAP excluded.

- Amsterdam II Criteria

  At least 3 family members with a HNPCC cancer (colorectal, endometrial, small bowel, ureter, or renal pelvis); one is a first degree relative of the other two; two consecutive generations represented; at least one individual younger than 51 years at diagnosis; FAP excluded.

- Age and Cancer Modified Amsterdam Criteria

  At least 3 family members with a OFCCR HNPCC cancer (colorectal, endometrial/uterine, gastric, small bowel, gastroesophageal, liver, pancreas, biliary duct, hepato-biliary, ovarian, kidney, ureter, brain, lymphoma); one is a first degree relative of the other two; two consecutive generations represented ; at least one individual younger than 61 years at diagnosis; FAP excluded.

INTERMEDIATE RISK

1. Proband and two relatives with any of the following cancers: colorectal, endometrial/uterine, gastric, small bowel, gastroesophageal, liver, pancreas, biliary duct, hepato-biliary, ovarian, kidney, ureter, brain, lymphoma AND 2 of the 3 are 1st degree relatives.

2. Proband and any family member with one of the above ca ≤ 35 years.

3. Proband ≤ 50 and relative with CRC ≤ 50 (1st and 2nd degree relative only).

4. Proband ≤ 35 years.

5. Proband with multiple primary CRC.

6. Proband with other primary HNPCC cancer listed above.

7. Proband meets at least 1 pathologic criterion: Multiple primary CRC, multiple adenomas (≥ 5), inflammatory bowel disease (IBD) Ulcerative colitis (UC), Crohn's disease, active chronic colitis, colitis-associated neoplasia, or other (concurrent or previous) cancer.

LOW RISK

- Proband does not meet high or intermediate risk categories

2.342 HNPCC Cancers

According to the Amsterdam criteria, HNPCC cancers are CRC, cancer of the endometrium, small bowel, ureter, or renal pelvis. For the Age and Cancer Modified Amsterdam Criteria, and the intermediate criteria, HNPCC cancers (recommended by the OFCCR) include cancers of the endometrium, stomach, small bowel, gastroesophagus, liver, pancreas, biliary duct, hepato-biliary system, ovary, kidney, ureter, brain, and lymphoma. In this study the above cancers, in all risk criteria, were classified as HNPCC cancers. However, for the low risk criteria, it is unlikely that these cancers are HNPCC cancers.

2.343 Cancer Diagnosis in Relatives

All reported family cancers were used in this analysis. An attempt was made to consent family members and examine medical records to confirm reported cancers. We also used the NCTRF as a resource to confirm reported cancers. Information including name (first, last, maiden), age, date of birth and residence of family members was used to search the registry.

2.344 Non-familial

A proband was considered to be non-familial or sporadic if he/she did not meet the high risk criteria or the intermediate risk criteria for HNPCC.

2.345 Informative vs. Uninformative Families

We developed a system to categorise families as either informative or uninformative. Informative families were defined as those having six relatives at risk in two consecutive generations for whom we had confirmed or unconfirmed medical history. Parents and their siblings must have reached age 60 or developed a HNPCC cancer. Uninformative families were defined as those failing to meet the informative criteria.

2.4 Laboratory Study

Pathology specimens (normal and tumour tissue) from probands were obtained for examination by microsatellite (MSI) analysis and immunohistochemistry (IHC) analysis. Normal tissue served as a control. If the participant was willing, a blood sample was drawn for study at a later date.

2.41 Study Population

2.411 Inclusion Criteria

Participants were included in the molecular study after tissue samples were reviewed by principle investigator Dr. Des Robb, a pathologist at Memorial University Hospital. Carcinoma arising within a polyp was included only if there was invasion into the stalk.

2.412 Exclusion Criteria

Cases were excluded if tumour was not resected, or the tissue sample was not appropriate (e.g. not enough tumour, multiple polyps in the same block, non-invasion in the sample).

2.413 Retrieval of Pathological Specimens

All Pathology reports identifying CRC cases occurring in the 1997 and 1998 calendar years were requested from the Health Sciences Centre, St. Clare's Mercy Hospital, The Salvation Army Grace General Hospital, and Carbonear General. They were then sorted by age categories: 20-69, and the remaining. Each report was reviewed carefully to ensure that the case was an adenocarcinoma. The meditech system was then used to eliminate all cases that did not reside on the Avalon Peninsula at the time of their diagnosis (n= 39). A final list was sorted and submitted to each of the hospitals. Paraffin blocks containing formalin-fixed tumour and normal tissue, removed at the time of surgery, were collected.

2.42 Primary Outcome Molecular Study

MSI analysis and IHC analysis were performed on the tissue of each proband.

2.421 Microsatellite Analysis

The normal and tumour DNA from each patient's tissue was compared for MSI at a total of five microsatellite markers, including two mononucleotide markers BAT 26, BAT 25, and three dinucleotide markers D2S123, D17S250 and D5S346. These are well

characterized markers known to be very informative when testing for MSI, and are the accepted panel of markers described in literature for MSI analysis of CRC (Boland et al, 1998). DNA was extracted from the tumour and normal blocks, using the Qiagen DNeasy Tissue Kit (cat # 69504) (Appendix G). PCR reactions were carried out on the DNA for each of the markers used. The PCR products were run on acrylamide gels.

Initially each case was examined by two independent investigators, Ms. Angela Hyde and Dr. Ban Younghusband. When it was confirmed that both investigators were reaching the same conclusions Angela Hyde continued this process alone. If there were any questionable results then Dr. Younghusband assessed the situation and if necessary the procedure was repeated. Each case was described as either MSI-high ($\geq$ 30% markers demonstrate instability) MSI-low ($<$ 30% demonstrated MSI), or MSS (no markers exhibited MSI) after results had been obtained for each of the five markers (Boland et al 1998,; Thibodeau et al 1998.) MSI-high indicates that at least 40%, or two of the five, markers examined showed instability in the tumour DNA. MSI-low indicates that only one of the five markers show instability. When this result was obtained a second set of markers was used, including Bat 40, D17S787, D18S58, D20S100, and D7S519. The final description of MSS indicates microsatellite stability, whereby none of the five markers examined show instability in the tumour DNA. MSI-L tumours were reported as MSS for the purpose of this project. Loss of Heterozygosity (LOH) was also noted. LOH was noted if an allele was absent from the tumour but present in the normal tissue and the tumour was considered to be MSS.

2.422 Immunohistochemistry (IHC) Analysis

Microscope slides were prepared of formalin-fixed paraffin-embedded tissue containing approximately 50% tumour and normal tissue. The tissue was stained by immunohistochemistry using antibodies against three proteins MSH2, MLH1, and MSH6. These proteins are coded by mismatch repair genes that are responsible for over 90% of mutations leading to HNPCC. Large Volume DAKO LSAB and System method was used for staining with the MLH1 and MSH6 antibodies. The DAKO ENVISION method was used to stain for the MSH2 antibody. These staining procedures are sensitive, versatile, and enable the processing of numerous specimens in just over one hour (Dako instructions) (Appendix H).

Dr. Des Robb and Angela Hyde reviewed the slides. If the nucleus of the cell stained a brown color then the protein was present but if the nucleus failed to stain brown then the protein was absent. Two methods of grading the slides were developed. Intensity of the staining was determined by a 4 point scale: 0 = no stain; 1 = weak stain; 2= moderate stain; and 3 = strong stain. Frequency of staining (% positive tumour cells) was determined by a 5 point scale: 0= all cells negative; 1 = (1-10%); 2 = (11-50%); 3= (51-75%); and 4= (> 75%).

If a patient carries a mutation affecting the antibody epitope, such as a truncating mutation in one of these genes, the tumour will not express a full copy of the associated protein. In that case the antibody may not bind, and the gene containing the disease causing mutation can be determined. This determination will be of immense value when searching for the mutation itself.

2.5 Family History and Laboratory Study

Correlations were made comparing family history classification, MSI and IHC analysis. Correlations were also made between MSI analysis and tumour location.

2.51 Diagnostic Utility of the Tests

Sensitivity and specificity analysis for microsatellite classification for HNPCC cases was defined using family history as the gold standard. In this study sensitivity was defined as MSI-H by MSI analysis in high risk cases, and specificity was defined as MSS by MSI analysis in sporadic cases. Sensitivity and specificity analysis for laboratory methods for detection of HNPCC cases was defined using Family history as the gold standard. Sensitivity was defined as MSI-H or absence of expression of hMSH6 by IHC in high risk cases. Specificity was defined as MSS and expression of all protein expression by IHC in sporadic cases. Sporadic cases included informative families only.

2.6 Follow-up

A follow-up letter was sent to all probands and any participating family members. This letter explained all study outcomes. Each participant was informed of the family history risk assessment. They were not given the results of the lab tests. These tests were performed for research purposes and not clinical purposes. A referral to a geneticist was given to those individuals suspected of having a hereditary form of CRC.

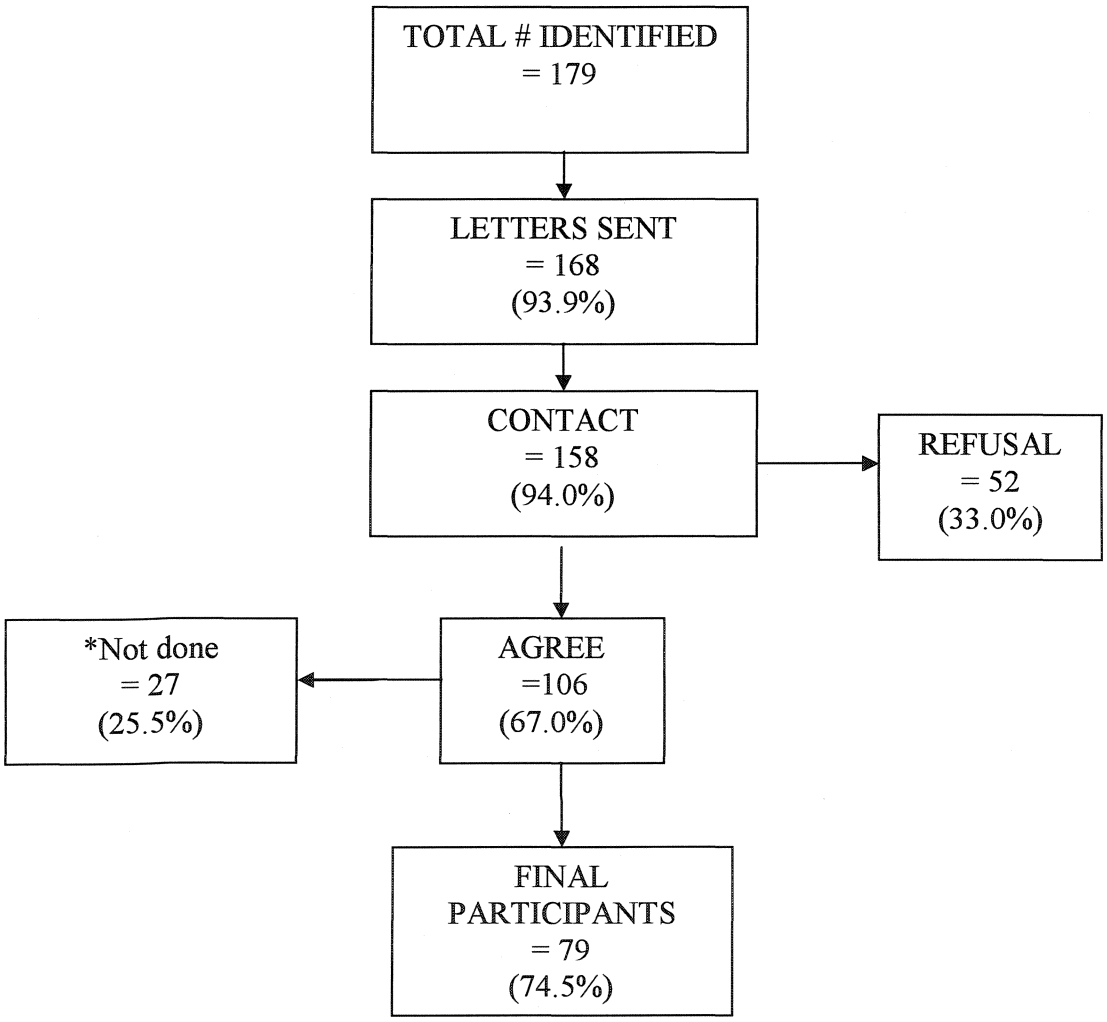
CHAPTER 3: RESULTS

3.1 Baseline Results

Proposal Development

179 incident cases of CRC on the Avalon Peninsula during 1997 and 1998 were identified. Figure 1 summarises the recruitment process.

FIGURE 1: Recruitment of study participants (n= 79)



*not done because of difficulties making contact. They are being followed up by the NFCCR.

After initial contact letters were sent out by treating physicians, 79 individuals participated and 52 opted out. There was contact with another 29, but their participation in the study has not been confirmed. There was no contact with 19 individuals.

3.12 Recruitment of Study Participants

The initial method used to identify study participants was the NCTRF tumour registry. This method identified 178 CRC cases. This group is representative of approximately half of the Newfoundland CRC cases for 1997 and 1998. Eight of the 178 cases were excluded: 4 were inappropriately diagnosed, 2 were recurrences, 1 appeared twice, and one did not live in the selected regions. All Pathology reports and discharge summaries of the St. John's Health Care Corporation (meditech) were also searched for CRC cases. There were 14 individuals identified who were not on the NCTRF list. These were reported to the NCTRF and they are now registered. Five of these cases were excluded on the basis that 4 were inappropriately diagnosed and the identity of one was questionable. The other nine were included in the study. Of the total eligible group of 179 (Table 1), 168 letters were sent out, and contact was made with 158 patients. Fifty-two patients declined to participate, 27 patients need further follow-up and 79 participated in the current study. Thus contact was made with 88.3% of eligible participants and 50.0% of those contacted enrolled in this current study.

TABLE 1: Identification of participants through the NCTRF, Meditech, and pathology reports

IDENTIFYING RESOURCE		
NCTRF	178 Identified	
		8 excluded - 4 inappropriate diagnosis - 2 recurrences - 1 appeared twice - 1 not in region
	170 APPROPRIATE	
MEDITECH	6 Additional Identified	
		3 excluded – 2 inappropriate diagnosis - 1 questionable identity
	3 APPROPRIATE	
PATHOLOGY REPORTS	8 Additional Identified	
		2 excluded – 2 inappropriate diagnosis
	6 APPROPRIATE	
Total Selected for recruitment = 179		
NCTRF Recheck (Summer 2002)	8 out of 9 additional colorectal cancers identified through Pathology reports and meditech were registered with the NCTRF	

Of 79 study participants there were 65 probands and 14 proxies (Table 2). Ten proxies were identified because the proband was deceased. Two were identified as the most appropriate contact to provide a detailed family history and two were identified as guardians. Diagnosis was in 1997 in forty-one cases and in 1998 in 38 cases. The total included 34 females (43%) and 45 male probands (57%). The mean age of CRC diagnosis was 56.1 years, and the age range of CRC diagnosis was 36-69 years of age. Twenty-six of these cases occurred in the proximal colon, 45 occurred in the distal colon, and the location of the remaining (n=8) was not specified. The participants resided in a total of 30 towns on the Avalon Peninsula within Health Boards 1 and 2. Sixty-one individuals resided in Health Board 1, and 18 individuals resided in Health Board 2 at the time of their diagnosis. Parents and grandparents originated from many different areas of the province (n= 124) or from outside of Newfoundland (n= 18).

A t-test was conducted to determine if there was a difference in age for participants and non-participants. Pearson chi-squared test was conducted to determine if there was difference in either gender or tumour location for participants and non-participants. There was no significant difference between participants and non-participants for age of diagnosis ($p= 0.48$), gender ($p= 0.51$), and tumour location ($p= 0.60$). This would suggest that the group studied is representative of the whole population for these factors.

Table 2: Characteristics of Probands (n = 79) and of Non-Participants (n = 100)

	PROBANDS	NON-PARTICIPANTS
Mean Age (yrs) colorectal cancer diagnosed	56.1	56.6
Median age (yrs)	57	58.0
Age Range	33 (36-69)	44 (25-69)
Male	45 (57%)	52 (52.0%)
Female	34 (43%)	48 (48.0%)
Dead	11 (13.9%)	46 (46.0%)
Tumor Site		
Proximal colon	26 (32.9%)	39 (39.0%)
Distal colon	45 (57.0%)	53 (53.0%)
Not specified	8 (10.1%)	8 (8.0%)
Diagnosis Year		
1997	41 (51.9%)	54 (54.0%)
1998	38 (48.1%)	46 (46.0%)
Newfoundland Health Board		
Health Care Corporation St. John's	61 (77.2%)	74 (74.0%)
Avalon Health Board	18 (22.8%)	26 (26.0%)

3.13 Data Collection

Family history was collected from 79 study participants. Sixty-eight study participants were interviewed in person either at the Health Sciences Centre, Carbonear General, or in the individual's home. Two individuals reported their medical family history by standardised mail-in form. One chose to conduct a telephone interview. Another eight requested that family history previously obtained by the Medical Genetics program be used. Medical records from the NCTRF and hospitals were collected on study participants and some of their affected family members. The collection of family medical records was difficult and will be continued, where necessary, by a genetic counsellor.

Twenty-six families were previously identified through the Medical Genetics program at the Health Sciences Centre in St. John's, Newfoundland. Twenty probands were seen themselves, and for 6 families another family member had been seen. Dr. Jane Green was responsible for the collection of family history and clinical data from these families. The reasons for referral to Medical Genetics program varied but most were to rule out a hereditary cancer. For 12 study participants the Medical Genetics file were reviewed after initial interview and after permission was given. Eight participants gave permission to access their chart initially so that a repeat of the information was not necessary. Six participants were not aware that they family history was previously collected by the Medical Genetics program. It was on review of the study pedigrees that Dr. Green recognised these 6 families.

3.2 Family History Study

3.21 Risk Classification

Three generation family histories were collected from all study participants. Two study participants (#11, #12) are first cousins and are therefore considered to have the same risk for HNPCC. They were included in the analysis only once. Therefore there were a total of 78 families for risk analysis (Table 3).

Table 3: HNPCC Risk Classification for 78 families

	N	% Total
HIGH (AC I)	6	7.7
HIGH (AC II)	1	1.3
Age and Cancer Modified AC (included families in which youngest cancer was 51-60, OFCCR HNPCC cancers)	15	19.2
INTERMEDIATE 1 (no age limit, 2 of 3 affected are 1st degree, any OFCCR HNPCC cancer)	14	17.9
INTERMEDIATE 2 (Relative with CRC $\leq$ 35)	1	1.3
INTERMEDIATE 5 (Double primary)	3	3.8
INTERMEDIATE 7 (pathology)	4	5.1
LOW	34	43.6
Total	78	

AC -Amsterdam Criteria

Twenty-two families (28.2%) were considered High Risk (HR) for HNPCC. Six of these families (27.3%) fulfilled the Amsterdam Criteria I (AC I), 1 family (4.5%) fulfilled the Amsterdam Criteria II (AC II), and 15 families (68.2%) fulfilled the Age and Cancer Modified Amsterdam Criteria.

Twenty-two families (28.2%) were considered Intermediate Risk (IR) for HNPCC. Fourteen of these probands (63.6%) fulfilled the intermediate criteria based on having 2 relatives with any of the HNPCC cancers (intermediate 1 (IR-1)). One proband (4.5%) fulfilled the intermediate criteria based on having a relative with colon cancer $\leq$ 35 years old (IR-2). Three probands (13.6%) fulfilled the intermediate criteria based on having had multiple primary colon cancers (IR-5). Four probands (18.2%) fulfilled the pathological criterion for intermediate risk (IR-7) of which 3 (75.0%) had multiple adenomas as well as CRC and one (25.0%) had inflammatory bowel disease (IBD). No families fulfilled the intermediate criteria 3, 4, and 6.

Thirty-four families (43.6%) did not meet either the HR or IR criteria and were therefore considered Low Risk (LR) for HNPCC.

3.22 Family Informativeness

Of 34 low risk families, 21 (61.8%) were informative, and 13 (38.2%) were uninformative. Reasons for uninformative families included: less than 6 primary (siblings and parents) or secondary (aunts and uncles) relatives at risk (n= 3); less than six siblings at risk who were $\geq$ and 60 years old (n=3); insufficient family history (n=7).

3.23 Description of Cancers

Tables 4 through 8 summarize the occurrence of different types of cancer in each of the HR groups, the IR group1, and the LR study group. These tables show that CRC was the most common occurring cancer in the HR, IR, and LR families.

Fifteen different cancers were reported for the AC I group (Table 4). Stomach cancer was the most common extracolonic cancer. It occurred once in each of five families.

Five different cancers were reported for the AC II group (Table 5). Endometrial cancer was the most common extracolonic cancer, it occurred 2 times in this family.

Twenty-four different cancers were reported for the Age and Cancer Modified AC group (Table 6). Breast cancer was the most common extracolonic cancer occurring 17 times in 5 of 15 families. Twelve of the breast cancers occurred in the same family, a suspected hereditary breast cancer family (189). In this group stomach cancer was the most common HNPCC cancer occurring 13 times in 8 of 15 families.

Eighteen different cancer types were reported for the IR group 1 (Table 7). In this group stomach cancer was the most common extracolonic cancer occurring 13 times in 9 of 14 of the IR families.

Seventeen different cancer types were reported for the LR group (Table 8). Breast cancer (n=34) was the most common extracolonic cancer in the LR group. Eleven of the breast cancers occurred in the same family, a suspected hereditary breast cancer family (116).

Table 4: Cancers in Amsterdam Criteria I Families (n=6)

CANCER	Family 7				Family 11				Family 19				Family 35				Family 110				Family 156			
	#	Age Min	Age Max	Age Mean	#	Age Min	Age Max	Age Mean	#	Age Min	Age Max	Age Mean	#	Age Min	Age Max	Age Mean	#	Age Min	Age Max	Age Mean	#	Age Min	Age Max	Age Mean
Colorectal	5	28	57	44.2	7	28	49	39.1	4	47	64	56.0	5	44	81	60.6	4	48	69	60.0	4	39	66	52.5
Endometrium	0	-	-	-	2	40	43	41.5	0	-	-	-	0	-	-	-	0	-	-	-	0	-	-	-
Stomach	1	84	84	84.0	1	68	68	68.0	1	53	53	53.0	1	40			0	-	-	-	1	?	?	?
Ovary	0	-	-	-	0	-	-	-	0	-	-	-	0	-	-	-	0	-	-	-	0	-	-	-
Pancreas	1	44	44	44.0	0	-	-	-	0	-	-	-	0	-	-	-	0	-	-	-	0	-	-	-
Liver	0	-	-	-	0	-	-	-	1	53	53	53.0	0	-	-	-	0	-	-	-	0	-	-	-
Kidney	1	36	36	36.0	0	-	-	-	0	-	-	-	0	-	-	-	0	-	-	-	0	-	-	-
Bladder	1	54	54	54.0	0	-	-	-	0	-	-	-	0	-	-	-	0	-	-	-	0	-	-	-
Uterus	1	43	43	43.0	0	-	-	-	0	-	-	-	1	56	56	56.0	0	-	-	-	0	-	-	-
Lung	0	-	-	-	0	-	-	-	-	-	-	-	1	40	40	40.0	0	-	-	-	0	-	-	-
Leukemia	0	-	-	-	0	-	-	-	0	-	-	-	1	62	62	62.0	0	-	-	-	0	-	-	-
Cervical	0	-	-	-	0	-	-	-	1	46	46	46.0	0	-	-	-	0	-	-	-	0	-	-	-
Breast	1	67	67	67.0	0	-	-	-	0	-	-	-	0	-	-	-	0	-	-	-	0	-	-	-
Coccyx	1	44	44	44.0	0	-	-	-	0	-	-	-	0	-	-	-	0	-	-	-	0	-	-	-
Multiple Myeloma	0	-	-	-	0	-	-	-	0	-	-	-	2	40	83	61.5	0	-	-	-	0	-	-	-

Table 5: Cancers in Amsterdam II Criteria Family (family 122)

CANCER	#	Age Min	Max	Mean
Colorectal	2	34	36	35
Endometrial	2	37	40	38.5
Ovary	1	40	40	40.0
Oesophagus	1	43	43	43.0
Tongue	1	60	60	60.0
Unknown	1	41	41	41.0

*age unknown

Table 6: Cancers in Age and Cancer Modified Amsterdam Criteria Families (n=15)

CANCER	# of Families		Age		
			Min	Max	Mean
Colorectal	50 (*1)	15	45	94	61.9
Stomach	13 (*1)	8	50	89	69.1
Endometrial	2	1	38	58	48.0
Brain	2 (*1)	2	62	62	62.0
Ovary	3 (*1)	3	60	75	67.5
Pancreas	6	3	52	69	61.7
Small Bowel	1	1	54	54	54.0
Kidney	1	1	47	47	47.0
Gallbladder	1	1	58	58	58.0
Oesophagus	1	1	59	59	59.0
Uterus	2 (*1)	2	47	47	47.0
Breast	17 (*1)	**5	28	72	51.3
Prostate	3	3	64	73	67.7
Lung	7 (*1)	5	31	69	50.2
Bladder	1	1	64	64	64.0
Basal cell cancer	1	1	40	40	40.0
Ocular Melanoma	1	1	38	38	38.0
Testicular	2	1	2	35	19.0
Multiple myeloma	1	1	70	70	70.0
Cervix	3	3	31	67	47.7
Leukemia	1	1	13	13	13.0
Leg	1	1	15	15	15.0
Ear	1	1	85	85	85.0
Mouth	1	1	43	43	43.0
Unknown	3 (*2)	2	86	86	86.0

*age unknown

** one family with 12 breast cancers (family 189)

Table 7: Cancers in Intermediate Risk 1 families (n= 15)

CANCER	#	# of Families	Age		
			Min	Max	Mean
Colorectal	37 (*1)	14	43	81	60.6
Stomach	13 (*3)	9	49	81	64.0
Endometrium	3	2	57	83	72.7
Liver	2 (*2)	2	?	?	?
Lymphoma	1	1	78	78	78.0
Gastroesophagus	1	1	52	52	52.0
Bladder	1 (*1)	1	?	?	?
Breast	10 (*5)	7	56	93	66.4
Prostate	9	7	50	75	62.2
Lung	5 (*1)	4	57	78	67.0
Lip	1 (*1)	1	?	?	?
Throat	3	3	40	69	55.0
Melanoma	1	1	54	54	54.0
Cervix	3	3	36	74	57.0
Basal cell	1	1	87	87	87.0
Bone	1	1	76	76	76.0
Leukemia	1	1	61	61	61.0
Skin	4 (*1)	3	56	65	60.3
Unknown	8 (*5)	5	79	87	83.0

*age unknown

Table 8: Cancers in Low Risk Families (n=34)

CANCER	#	# of Families	Age		
			Min	Max	Mean
Colorectal	46	34	36	80	57.0
Stomach	6	6	50	75	64.8
Ovary	1	1	59	59	59.0
Pancreas	2	2	49	72	60.5
Brain	1	1	60	60	60.0
Kidney	2	2	50	66	58.0
Lymphoma	1	1	78	78	78.0
Prostate	13 (*3)	9	60	92	76.8
Lung	5	4	49	72	58.4
Breast	34 (*1)	**15	30	87	59.1
Leukemia	4	3	25	78	50.5
Throat	3 (*1)	2	55	88	71.5
Cervix	1	1	65	65	65.0
BCC	1	1	63	63	63.0
Melanoma	1	1	55	55	55.0
Skin	2	1	50	58	54.0
Bone	2	2	55	72	63.5
Unknown	13 (*2)	11	45	92	65.1

*age unknown

** one family with 11 breast cancers (family 116)

3.24 Confirmed HNPCC Cancers

The NCTRF tumour registry and the Newfoundland and Labrador Medical Genetics clinic files were searched to confirm reported cancers. Table 9 shows that these resources confirmed 207 of 404 (51.2%) reported cancers. Thirty-two cancer cases could not be confirmed because the individuals did not reside in Newfoundland. Twenty-one of the 207 cancers (10.1%) were reported incorrectly which resulted in a change in a classification for two families (families 7 and 35, both HR).

Table 9: Confirmed cancers NCTRF and Medical Genetics Program for All Risk categories

	Low		Inter		High	
	#	%	#	%	#	%
Total NCTRF Searched	82	-	116	-	53	-
Total Confirmed	23	28.0	44	37.9	16	30.2
Total Med Genetics Searched	19	-	18	-	148	-
Total Confirmed	11	57.9	10	55.6	103	69.6
			N		%	
***TOTAL SEARCHED**			404		-	
TOTAL CONFIRMED			207		51.2	

*Total searched excludes those out of province

3.25 Previously Identified Families by Medical Genetics Program

Twenty-one families were previously seen for suspected hereditary CRC (Table 10). Two probands were identified as part of the Newfoundland "family C"; a previously recognised Newfoundland HNPCC family with a known hMSH2 germ-line mutation. The proband (#122) is positive for the mutation. Her pedigree drawn suggests that her family is at HR for HNPCC. The second proband (#115) was part of a branch of the "family C". Her immediate pedigree does not suggest that she is at risk for HNPCC. Testing for the "family C" mutation is currently on going for this individual. Three families (#'s116, 180,189) were previously seen for suspected hereditary breast cancer. One proband (#112) was recognised to be related to individuals with Bardet-Biedl (BBS) syndrome. Another proband (100) was recognised as a relation to a rod cone dystrophy family.

Table 10: Families previously referred to Medical Genetics (n= 26)

	N	% Total
Referred previously to Medical Genetics		
Proband	20	25.3
Other family member	6	7.6
Not referred	53	67.1

	N
Reason for Previous referral	
Colorectal Cancer	21
Breast Cancer	3
Bardet-Biedel syndrome	1
Other	1

3.26 Non-Participants and Those Previously Referred to Medical Genetics

Due to limitations in the design of the study it was not possible to collect the reasons for non-participation. The opt-out plan consisted of two options. Probands who received letters and wished to opt-out were to call their family doctor or the research team where an answering machine was available.

Ten individuals who opted out were identified to be under the care of the Medical Genetics department (Table 11). One individual was a member of the "family C". Another is currently being tested for Peutz-Jegher syndrome. Three were deceased, two of whom met intermediate risk criteria for HNPCC, and it is Dr. Green's opinion that one of the deceased would have participated in this study. Another was a member of a breast/ovarian cancer family, another had 2 primaries and therefore would be intermediate risk, another was definitely at a low risk for HNPCC and the other was diagnosed at a young age.

Table 11: Non-Participant Families Previously Referred to Medical Genetics (n= 10)

	N
Referred previously to Medical Genetics	10
Colorectal cancer	9
(Family C)	(1)
Breast/ Ovarian	1

3.3 Laboratory Study

3.31 Microsatellite Instability Analysis

Microsatellite instability analysis was completed for 74 of 79 cases. Analysis was not possible for two individuals due to an inappropriate sample. Three are pending. Sixteen cases (21.6%) had high frequency microsatellite instability (MSI-H) tumours. Fifty-eight cases (78.4%) had microsatellite stable (MSS) tumours. Five of the 58 MSS cases had low microsatellite instability (MSI-L) tumours. LOH occurred in 2 MSS cases (Table 12).

Table 12: Microsatellite Analysis Results for 74 cases

	N	%	% Total
Complete			
High Microsatellite instability	16	21.6	20.3
*Microsatellite stable	58	78.4	73.4
TOTAL	**74**		
Incomplete			
In progress	3		3.8
No sample	2		2.5
Total	79		

*Low microsatellite instability = 5/58

3.32 Immunohistochemistry Analysis

Immunohistochemistry analysis (IHC) was completed for 71 of 79 (89.9%) cases. Six cases are pending. Analysis was not possible for two cases due to an inappropriate sample. Sixteen cases (22.5%) had negative expression for one of the three proteins: seven cases (9.9%) are negative for hMLH1, two cases (2.8%) are negative for hMSH2, four cases (5.6%) were negative for hMSH6, and three cases (4.2%) were negative for hMSH2 and hMSH6. Fifty-five cases (77.5%) had positive expression of all three proteins (Table 13).

Table 13: Immunohistochemistry Analysis Results for 71 cases

	N	%	% Total
Complete			
Protein Absence			
hMLH1 negative	7	9.9	8.9
hMSH2 negative	2	2.8	2.5
hMSH6 negative	4	5.6	5.1
hMSH6 and hMSH2 negative	3	4.2	3.8
Protein expression	55		
TOTAL	**71**		
Incomplete			
Pending	6		
No sample	2		
Total	**79**		

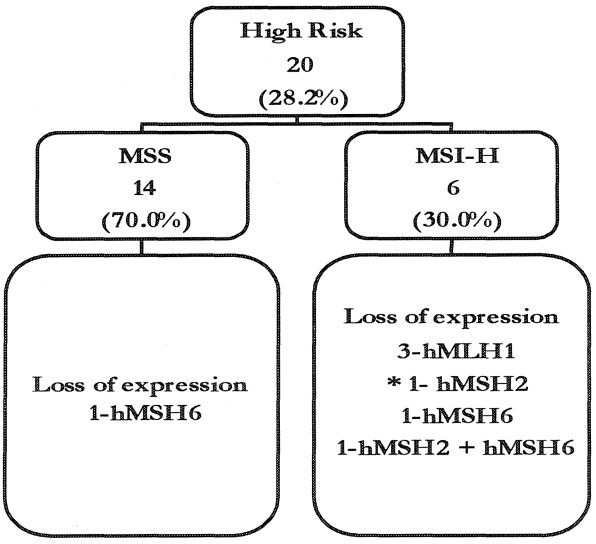
3.4 Family Risk and Molecular Correlation

There is family risk and molecular data for 71 cases. Two cases are members of the same family therefore were only included once.

HIGH RISK

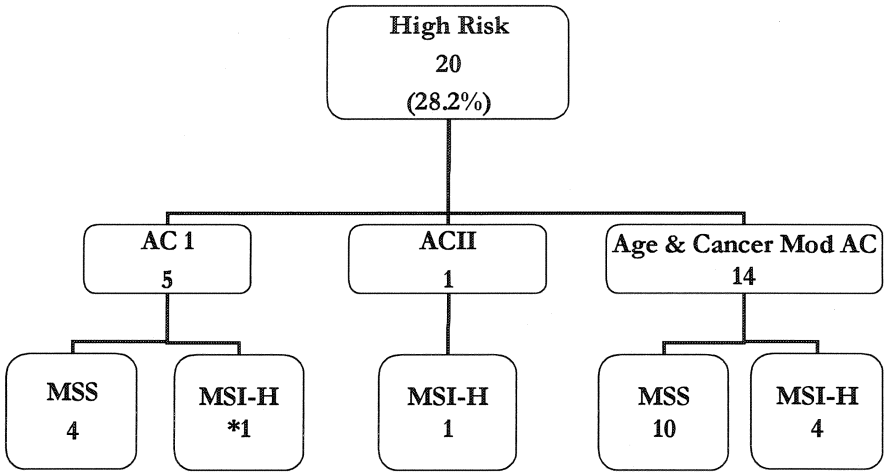
Twenty cases, high risk for HNPCC with their molecular data, were completed (Figure 2 & Figure 3). Analysis for five out of six cases fulfilling the Amsterdam criteria I were completed: one case (1.4%) was MSI-H and negative for hMSH2, another case (1.4%) was MSS and negative for hMSH6, and three cases (4.3%) were MSS and positive for all proteins (Figure 3). The case (7.0%) fulfilling the AC II was MSI-H and negative for both the hMSH2 and hMSH6 proteins. Fourteen of the 15 Age and Cancer Modified AC cases were completed: four cases were MSI-H of which 3 were negative for hMLH1 and one was negative for hMSH6, ten cases were MSS and positive for all proteins.

Figure 2: Family and Molecular Data for High Risk Families (n=20)



* Represents 2 probands who are members of the same family

Figure 3: Family and Microsatellite Analysis for 20 Families



* Represents 2 probands who are members of the same family

INTERMEDIATE RISK

Eighteen cases intermediate risk for HNPCC with molecular data were completed (Figure 4 and Figure 5). Ten cases (14.1%) of the Intermediate 1 criteria are complete: two cases (2.8%) were MSI-H of which one was negative for hMLH1 and the other was negative for hMSH2 and hMSH6, eight cases (11.3%) were MSS and positive for all proteins. The one case (1.4%) in the intermediate 2 criteria was MSI-H and negative for hMSH2 and hMSH6 proteins. Three cases (4.2%) were complete for the intermediate 5 criteria of which one case was MSI-H and negative for the hMLH1 protein and the two MSS cases were positive for all proteins. The 4 completed cases in the intermediate 7

criteria were MSS and positive for all proteins. Four intermediate cases (5.6%) do not have IHC analysis completed: two cases are microsatellite stable and two are incomplete for microsatellite analysis.

Figure 4: Family and Molecular data for Intermediate Families (n= 18)

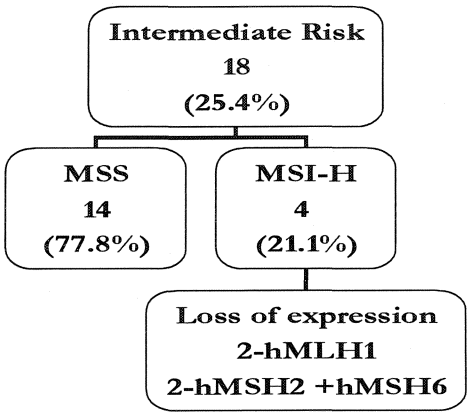
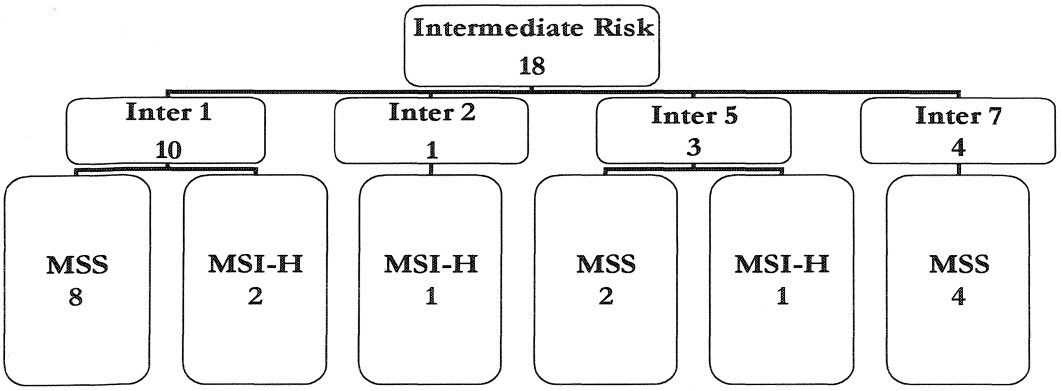


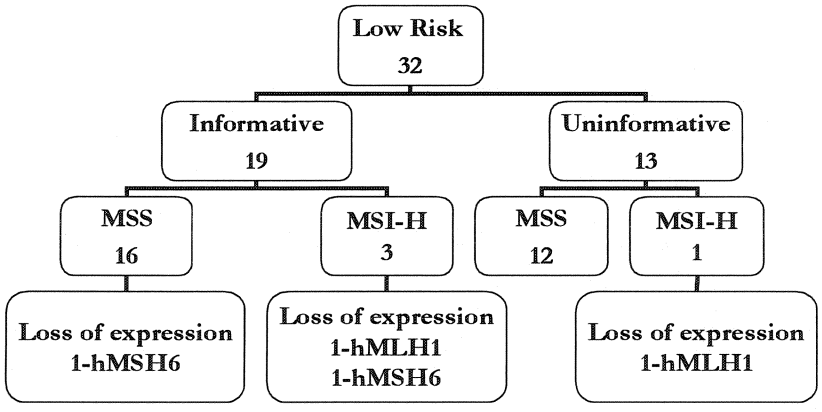
Figure 5: Family and Microsatellite Analysis for 18 Families



LOW RISK

Thirty-two of 34 (94.1%) low risk cases (45.1% of 71 families) had laboratory results completed, but only nineteen of these (59.4%) were from informative families (Figure 6). Of 19 informative cases, 3 were MSI (15.8%) and 16 were MSS (84.2%). For the informative MSI cases the hMLH1 protein was negative in one case, the hMSH6 protein was negative in another, and no loss of protein was observed in the 3rd case. Another informative case was MSS with negative expression of the hMSH6 protein. Only one of the 13 uninformative cases (7.7%) was MSI and it was negative for the hMLH1 protein.

Figure 7: Family and Molecular Data for 32 Informative and Uninformative Low Risk Families.



3.5 Pathology and Microsatellite Instability Analysis Correlation

Twelve (75%) of 16 MSI-H tumours were located in the proximal colon, two (12.5%) were located in the distal colon, and for two MSI-H tumours (12.5%) the location was unknown. The proportion of MSI-H and MSS tumours at each tumour location is shown in Table 14. To determine if there is an association between location of tumour and microsatellite analysis a pearson chi-square test was conducted. There is a significant difference (p= 0.002) in tumour location between the MSI-H tumours and MSS tumours.

Table 14: Tumour Location and Microsatellite Instability Analysis for 74 cases

	Tumour Site					
	Proximal colon		Distal colon		Unspecified	
	N	**%**	**N**	**%**	**N**	**%**
MSI-H	12	46.2	2	4.9	2	28.6
MSS	14	53.8	39	95.1	5	71.4
Total	**26**		**41**		7	

3.6 Diagnostic Utility of Methods

The sensitivity of microsatellite instability analysis in predicting cases that are high risk for HNPCC was 30.0% (Table 15). The specificity of microsatellite instability analysis in predicting cases that do not have HNPCC was 84.2%. The proportion of cases that had MSI-H tumours and that have HNPCC (positive predictive value) was 66.7%, and the proportion of cases that had MSS tumours that do not have HNPCC (negative predictive value) was 53.3%.

Table 15: Sensitivity and Specificity Analysis for Microsatellite Analysis Identifying HNPCC (n=38 families)

	HIGH	LOW	TOTAL
MSI-H	6	3	9
MSS	14	16	30
Total	20	*19	39

***informative families**

Two tumours (one HR and one LR) were MSS and did not express the hMSH6 protein. The sensitivity of either laboratory methods (MSI-H by microsatellite analysis or loss of protein expression by IHC) in predicting cases that are high risk for HNPCC was 35.0% (Table 16). The specificity of the methods in predicting cases that do not have HNPCC was 78.9%. The proportion of cases that have a positive laboratory finding

(MSI-H or loss of protein expression) and that have HNPCC (positive predictive value) was 63.6%, and the proportion of cases that have negative laboratory results (MSS and expressed all proteins tested) and do not have HNPCC (negative predictive value) was 53.6%.

Table 16: Sensitivity and specificity analysis for laboratory results diagnosing HNPCC (n=38 families)

	High	Low	Total
MSI-H or hMSH6-	7	4	11
MSS + All proteins expressed	13	15	28
Total	20	*19	39

***informative families**

3.7 Family Descriptions

3.71 HIGH RISK (n= 22 families)

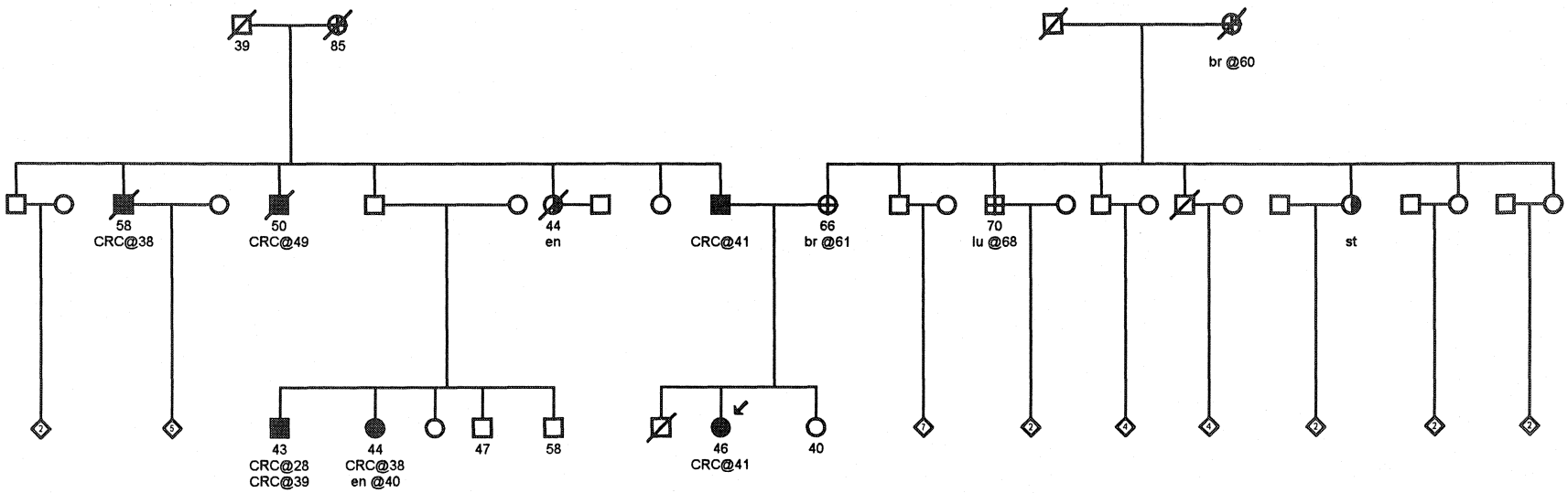
Amsterdam Criteria (n=6 families)

MSI-H and hMSH2- (n= 1)

Probands number 11 (pedigree 1) and 12 (pedigree 2) are first degree cousins. Proband number 11 is a 45 year old female who was diagnosed with a well-differentiated cancer of the cecum at age 41. Pathology revealed the presence of 4 polyps: 2 hyperplastic, 1 tubular adenoma, and one mucin producing. The colon cancer was identified upon screening recommended by the Dr. Green. She was born in St. John's where she now resides, and both parents and maternal and paternal grandparents also originated from St. John's. The pedigree constructed consists of 56 relatives. She has 4 first degree relatives: 2 siblings, and her parents. Her father was diagnosed with 2 HNPCC cancers: a CRC at age 41 and a stomach cancer at 68. He has also had a number of polyps (multiple tubular and villous adenoma) removed. Her mother was diagnosed with a breast cancer at age 61. There were no other cancers reported in first degree relatives. She has 17 second degree relatives. Four HNPCC cancers were reported; three in paternal relatives, and one in a maternal relative. A paternal aunt was diagnosed with endometrial cancer at age 43. A paternal uncle was diagnosed with CRC at age 38 and another paternal uncle was diagnosed with a CRC at age 49. A maternal aunt was diagnosed with a stomach cancer at age 55. Two non-HNPCC cancers were reported including a lung cancer in a maternal uncle and a breast cancer in her maternal grandmother. There were no other cancers in second degree relatives. Two paternal cousins (siblings) were each diagnosed with two HNPCC primaries. One cousin was

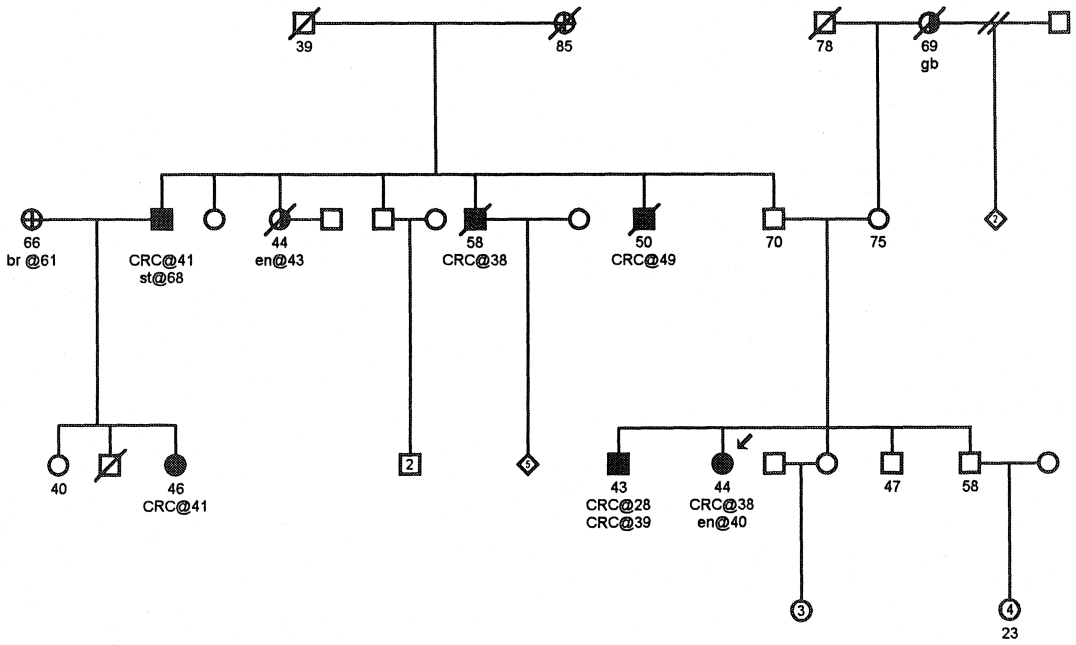
diagnosed with a CRC at age 28 and age 39. His sister was diagnosed with CRC at age 38 and endometrial cancer at age 40. This cousin is also a proband (12) in this study. There were no other cancers reported in this family. Both probands were previously referred to the Medical Genetics program.

Proband number 12 (pedigree 2) is a 44 year old female who was diagnosed, in 1997, with a well differentiated cancer of the cecum at age 38. This diagnosis was made on routine screening. Pathology also revealed the presence of a hyperplastic polyp. She was diagnosed with a second primary, an endometrial cancer, in 1999. She underwent a total hysterectomy and bilateral salpingo-oophorectomy. She was born in St. John's where she now resides. Her mother and her maternal grandparents originated from Harbour Grace. Her father and paternal grandparents originated from St. John's. The pedigree constructed consists of 35 relatives. She has 6 first degree relatives: 4 siblings, and her parents. Two HNPCC cancers were reported in her first degree relatives. Her brother was diagnosed with 2 CRC at age 28 and age 39. There were no other cancers reported in first degree relatives. Her mother is 75 and well. Her father is 71 and was reported to have had 6 polyps removed but records are not available, and whether he had subsequent screening is not known. Hyperplastic polyps were reported in 2 brothers. She has 19 second degree relatives. Three HNPCC cancers were reported in her second degree relatives. A paternal uncle was diagnosed with a CRC at age 49, another paternal uncle was diagnosed with a CRC at 41, and a third paternal uncle had CRC at 41 and stomach cancer at 68. A paternal aunt was diagnosed with an endometrial cancer at age 44. There were no other reported cancers in second degree relatives but her first cousin (proband 11) had a CRC at age 41.



Pedigree 1 High AC-Family 11

MSI
MSH2-



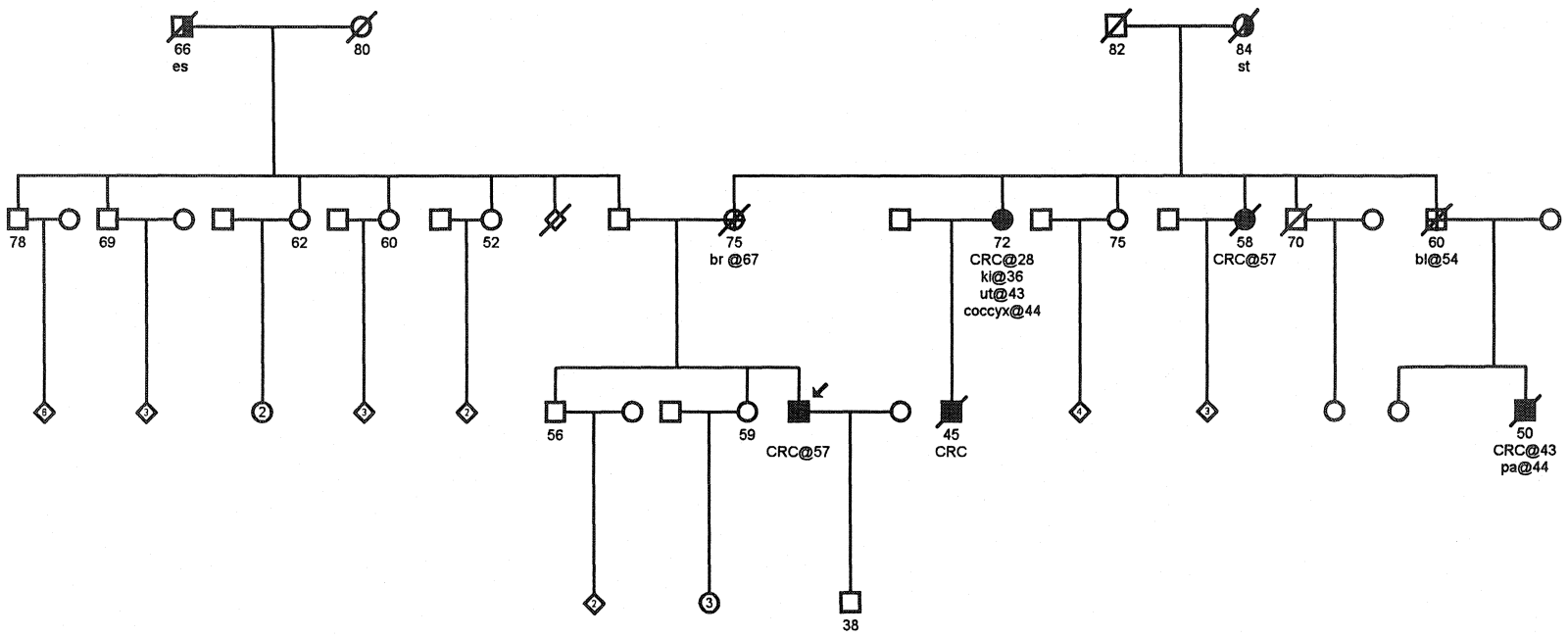
Pedigree 2 **High AC-Family 12**

MSI
MSH2-

MSS and hMSH6- (n= 1 family)

Proband number 7 (pedigree 3) is a 62 year old male who was diagnosed, in 1998, with a moderately differentiated cancer of the sigmoid colon. Pathology revealed the presence of one small polyp. He was born in St. John's where he now resides. His mother originated from Brookfield. His maternal grandparents originated form Bonnavista Bay North. His father and paternal grandfather originated from Hodge's Cove. His paternal grandmother originated from Winterton. The pedigree constructed consists of 52 relatives. He has 5 first degree relatives: 1 child, 2 siblings, and parents. His mother was diagnosed with a breast cancer at age 43 and died at 75 due to heart complications. Polyps were reported in his son at age 38 and in 2 siblings. No other cancers were reported in first degree relatives. He has 20 second degree relatives. A maternal uncle was diagnosed with a colon cancer at age 60. His paternal grandfather was diagnosed with an oesophageal cancer at age 66. A maternal cousin was diagnosed with a colon cancer at age 50. A maternal aunt was diagnosed with a cancer, type unknown at age 60. Review of the pedigree with Dr. Green identified that he was a member of a previously referred family to the Medical Genetics program. The proband did not appear to be aware of this. Seven HNPCC cancers were previously reported in his maternal relatives compared to his report of two HNPCC cancers. Some differences were noted. A maternal aunt was diagnosed with a CRC at age 28, a kidney cancer at age 36, a uterine cancer at age 43 and a coccyx cancer at age 44. The maternal uncle had bladder cancer at age 54 with extension to the colon. A maternal aunt was diagnosed with CRC at age 57. His maternal grandmother was diagnosed with stomach cancer (younger than age 84). No other cancers were reported in second degree relatives. A maternal cousin was

diagnosed with CRC at age 36 and another maternal cousin was diagnosed with a CRC at age 43 and a pancreatic cancer at age 44. Extension of his maternal grandfather's family indicates HNPCC cancers in other branches.

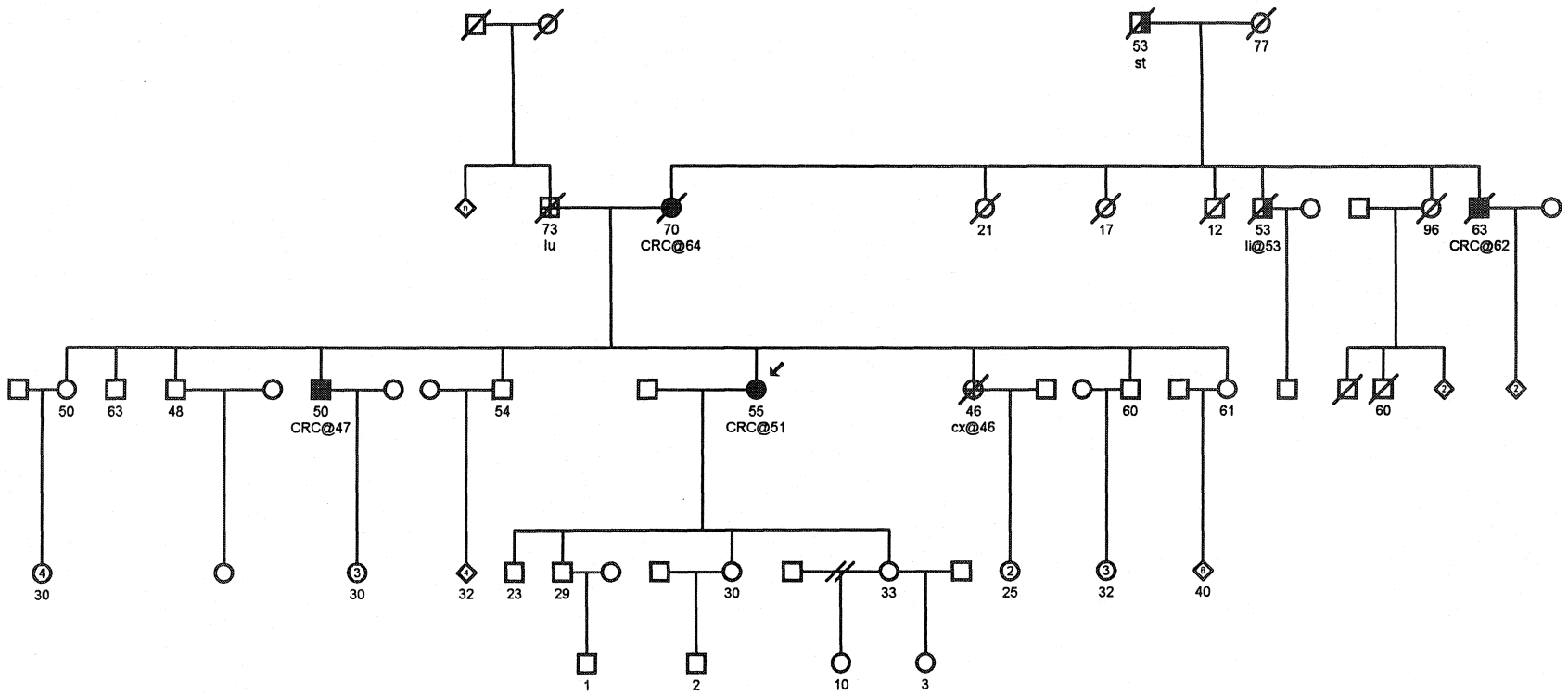


Pedigree 3 High AC-Family 7

MSS
MSH6-

MSS and no loss (n= 3 families)

Proband number 19 (pedigree 4) is a 56 year old female who was diagnosed, in 1998, with a well-differentiated cancer of the sigmoid colon at age 51. She was born Chance Cove, Trinity Bay where she now resides. Her mother and maternal grandparents originated from Chance Cove. Her father originated from Norman's Cove. Her paternal grandparents originated from Chance Cove. The pedigree constructed consists of 55 relatives. She has 12 first degree relatives: 4 children, 6 siblings, and parents. Two HNPCC cancers were reported in first degree relatives. Her mother was diagnosed with a CRC at age 64. She died at age 70 after the cancer had reoccurred. Her brother was diagnosed with CRC at age 47. Two non-HNPCC cancers were reported which include a lung cancer in her father and a cervical cancer in a sister. There were no other cancers reported in first degree relatives. One daughter and three siblings had colonoscopy investigations. She has 36 second degree relatives. Two HNPCC cancers were reported. A maternal uncle was diagnosed with CRC at age 62 and another maternal uncle was diagnosed with a liver cancer at age 53. There were no other cancers reported in this family. She was on the waiting list for the Medical Genetics program. Records are currently being retrieved by the department.

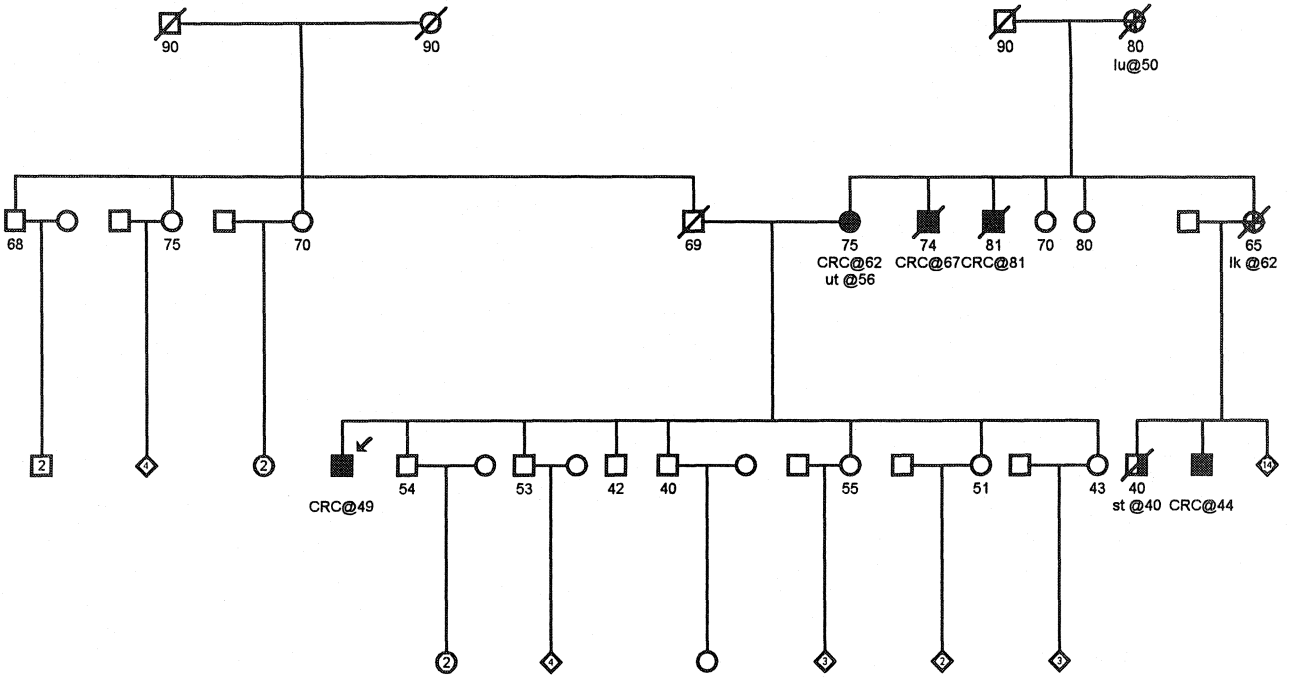


Pedigree 4 **High AC-Family 19**

MSS
no loss

Proband number 35 (pedigree 5) is 54 year old male who was diagnosed, in 1998, with a moderately differentiated cancer of the sigmoid at age 49. He had a recurrence a year later and has also been diagnosed with liver metastasis. He was born in Wesleyville, Bonavista Bay and now resides in St. John's. His mother and maternal grandparents originated from Griquet. His father and paternal grandparents originated from Pound Cove, Bonavista Bay. The pedigree constructed consisted of 59 relatives. He has nine first degree relatives: seven siblings, and parents. The proband reported that his mother was diagnosed with a uterine cancer at age 60, a maternal uncle was diagnosed with a leukemia, a maternal aunt was diagnosed with a cancer, type unknown, and his maternal grandmother was also diagnosed with a cancer, type unknown. There were no other cancers in this family as reported by the proband. Review of the pedigree with Dr. Green identified that the proband's family had previously been referred to the Medical Genetics program. Comparisons were made between the two pedigrees and a different story emerged. HNPCC cancers were previously documented. The proband's mother was diagnosed with uterine cancer at age 56 and also a cancer of the sigmoid colon at age 62. She is still alive. The maternal uncle that he had reported as having leukemia in fact had a CRC at age 67. A maternal aunt had leukemia at age 62. Another maternal uncle had a CRC at age 81 and a cancer of the cerebellum at age 83. Two maternal cousins were diagnosed with HNPCC cancers. They include a CRC at age 44 and a stomach cancer (age not given). His father died from kidney failure at age 69. Extension of the maternal grandmother's family identifies HNPCC in other branches of her family. The grandmother's sister had an astrocytoma at age 69, and her brother had a CRC at age 63. Another brother died at age 42 from heart complications. All three have offspring with

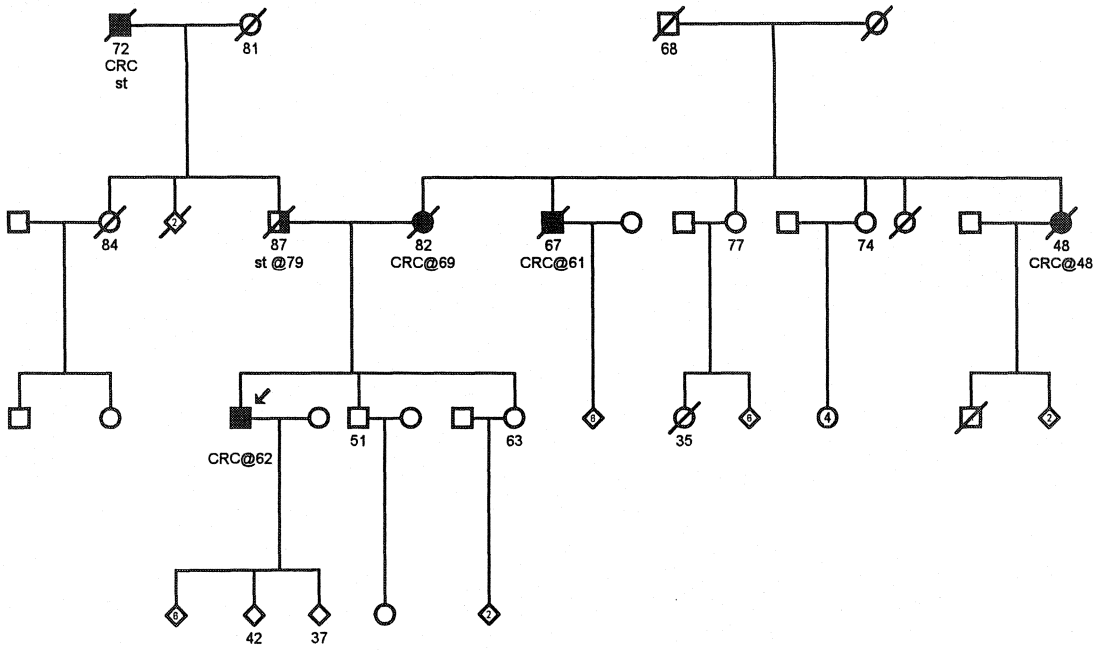
HNPCC cancers including CRC, liver, brain, and other possible HNPCC cancers including skin and transitional cell cancer of the bladder.



Pedigree 5 **High AC-Family 35**

MSS
no loss

Proband number 110 (pedigree 6) is a 67 year old male who was diagnosed, in 1998, with a moderately differentiated cancer of the rectosigmoid junction at age 62. Pathology revealed the presence of hyperplastic polyps. He was born in Harbour Deep, White Bay and now resides in St. John's. His mother and maternal grandparents originated from Harbour Deep, White Bay. His father and paternal grandmother originated from Fair Island, Bonavista Bay. His paternal grandfather originated form Swain's Island. The pedigree constructed consists of 51 relatives. He has 10 first degree relatives: 6 children, 2 siblings, and parents. His parents were both diagnosed with possible HNPCC cancers. His mother was diagnosed with CRC at age 69 and she died at the age of 83. His father was diagnosed with stomach cancer at age 79. No other cancers were reported in first degree relatives. One brother was scoped. The proband has 19 second degree relatives. Four HNPCC cancers were reported in his second degree relatives. A maternal aunt was diagnosed with CRC at age 61. Another maternal aunt was diagnosed with CRC at age 48. It was reported that his paternal grandfather was diagnosed with stomach and CRC and died at age 72. This proband was previously referred to the Medical Genetics program. Extension of the paternal grandfather's family identifies possible HNPCC in other branches of his family. Two of the grandfather's brothers were reported to have HNPCC cancers. One of these brothers had three sons who were reported to have CRC and stomach cancers.

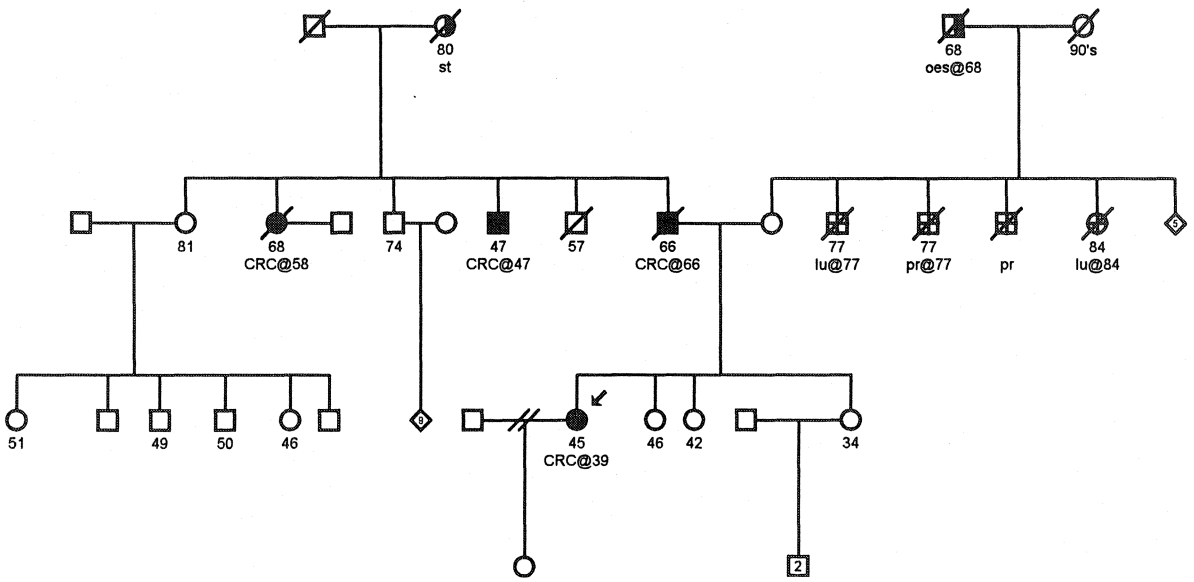


Pedigree 6 High AC-Family 110

MSS
no loss

No Sample Available for Molecular and Pathology Analysis (n= 1 family)

Proband number 157 (pedigree 7) is a 45 year old female who was diagnosed with a well-differentiated cancer of the rectosigmoid junction at age 39. Polyps were indicated (no details given). She was born in St. John's where she now resides. Her mother and maternal grandfather originated from Mobile, Southern Shore. Her maternal grandmother originated from Tors Cove. Her father and paternal grandfather originated from Little Paradise, Placentia Bay (PB). Her paternal grandmother originated from St. Leonard's, PB. The pedigree constructed consists of 78 relatives. She has 6 first degree relatives: 1 child, 3 siblings, and parents. Her father was diagnosed and died with a CRC at age 66. There are no other cancers reported in first degree relatives. Two of her sisters were scoped. She has 31 second degree relatives. Four HNPCC cancers were reported in second degree relatives. A paternal aunt was diagnosed with a CRC at age 58. A paternal uncle was diagnosed with a CRC at age 47. Her paternal grandmother was diagnosed with a stomach cancer. Her maternal grandfather was diagnosed with an esophageal cancer. Non-HNPCC cancers reported include two prostate cancers in paternal uncles, and lung cancer in a maternal uncle and maternal aunt. There were no other reported cancers in second degree relatives. Two paternal cousins (siblings) had tubular adenomas on screening scopes. This proband was previously referred to the Medical Genetics program. Extension of the paternal grandmother's family and the maternal grandmother's family identifies a history of cancers. There are no available records.



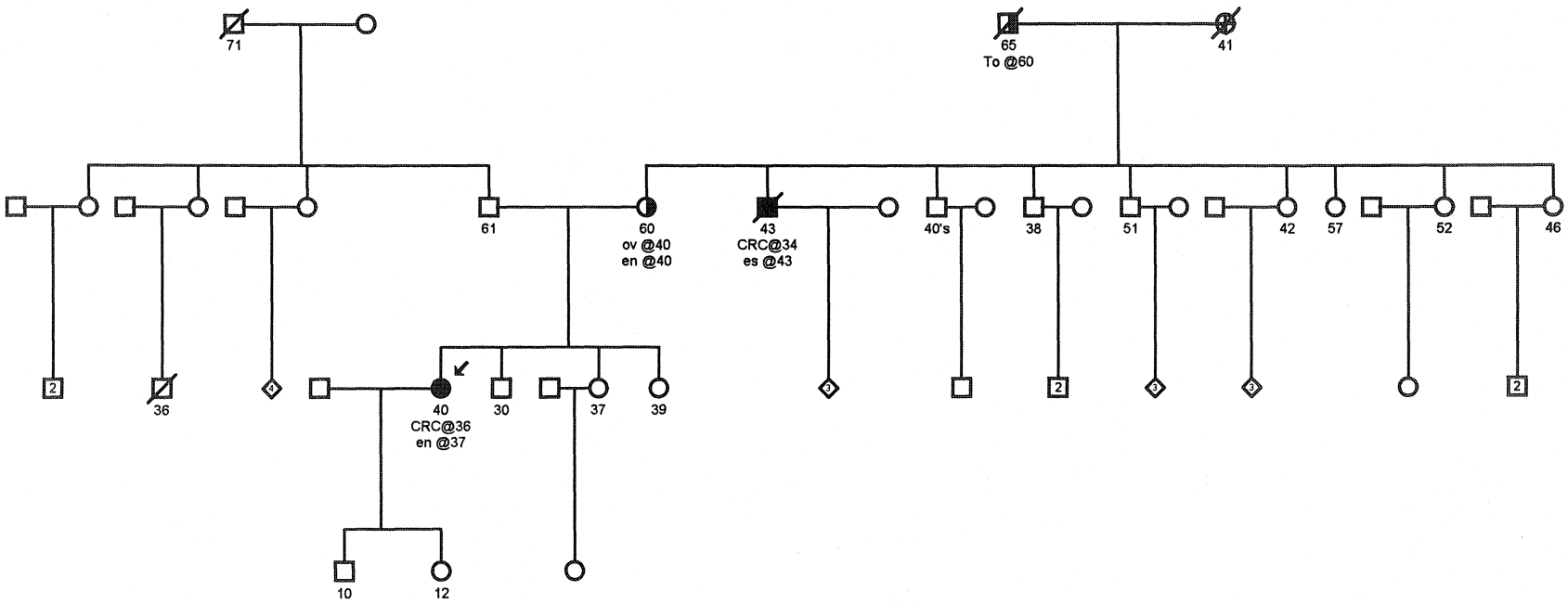
Pedigree 7 High AC-Family 157

No sample

Amsterdam II Criteria (n= 1 family)

MSI-H and hMSH2- and hMSH6- (n=1 family)

Proband number 122 (pedigree 8) is a 41 year old female who was diagnosed, in 1997, with a well differentiated cancer of the rectum at age 36. Pathology revealed the presence of a hyperplastic polyp. At age 37 she was diagnosed with a cancer of the endometrium. She was born on Bell Island. Her maternal grandparents originated from Pound Cove, BB. The pedigree constructed consists of 45 relatives. She has seven first degree relatives: 2 children, 3 siblings, and parents. Her mom was diagnosed with two HNPCC cancers, an ovarian cancer and an endometrial cancer at age 40. No other cancers were reported in first degree relatives. Three HNPCC cancers were reported in second degree relatives. A maternal uncle was diagnosed with a CRC at age 34 and an oesophageal cancer at age 43. Her maternal grandfather was diagnosed with a tongue cancer at age 60 and a HNPCC cancer, stomach cancer, at age 63. Her maternal grandmother died at age 41 of a cancer, type unknown. The proband is a member of the previously identified family ("Family C") which has a known mutation of hMSH2. The proband has been seen by the Medical Genetics program and she and three others in family 122 have tested positive for this mutation.



Pedigree 8 **High AC II-Family 122**

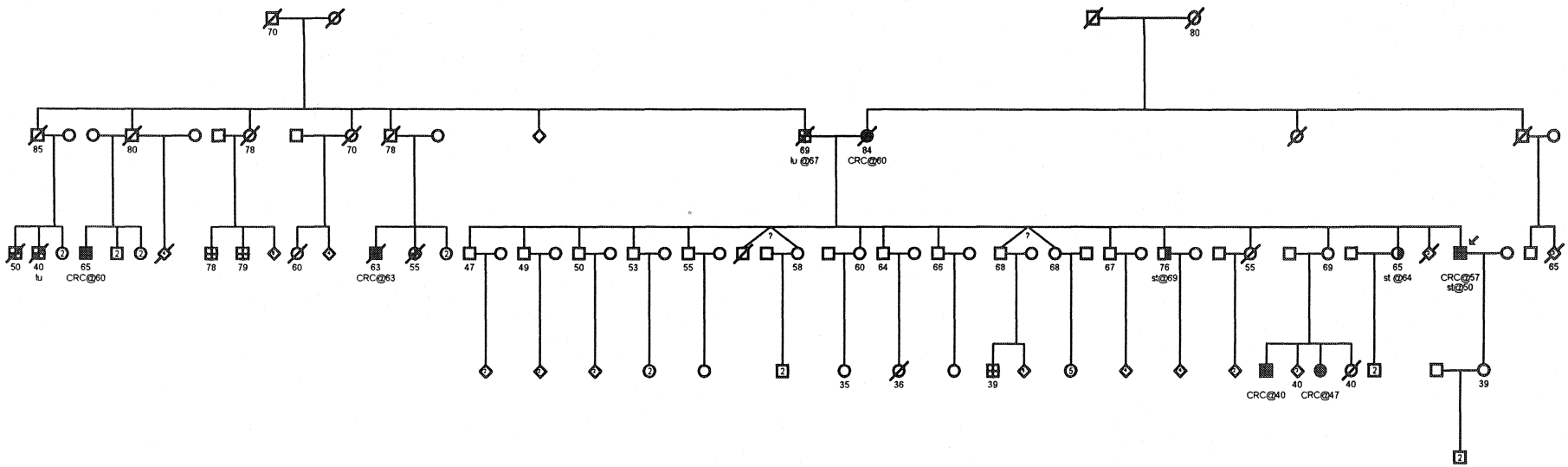
MSI
MSH2- and MSH6-

Age and Cancer Modified Amsterdam Criteria (n= 15)

MSI-H and hMSH6- (n= 1 family)

Proband number 17 (pedigree 9) is a 63 year old male who was diagnosed, in 1997, with a well differentiated cancer of the ascending colon at age 57. Chart review indicated three adenomatous polyps throughout the colon in 1997. He was also diagnosed with a stomach cancer at age 49. He was born in St. John's where he now resides. His mother and maternal grandmother originated from St. John's. The origin of his maternal grandmother was not given. His father and paternal grandparents also originated from St. John's. The pedigree constructed consists of 114 relatives. He has 23 first degree relatives: 1 child, 20 siblings, and parents. Three HNPCC cancers were diagnosed in first degree relatives. CRC was diagnosed in his mother at age 60 and she died at age 84. Stomach cancer was diagnosed in a sister at age 64 who died a year later, and also in a brother at age 69. Three siblings died at a young age from tuberculosis, another died just after birth and another died at age 55 from complications of rheumatoid arthritis. His daughter was diagnosed with ulcerative colitis at age 26 and two brothers have been diagnosed with Crohns disease. His father was diagnosed with lung cancer at age 67, and died at age 69. No other cancers were reported in his first degree relatives. He has 57 second degree relatives. Two HNPCC cancers, both CRC, were reported in a niece and a nephew (siblings) in their 40's. There were no other reported cancers in second degree relatives. There were a number of cancers reported in paternal cousins. Two HNPCC cancers, both CRC, were reported at age 63 and at age 65. Other cancers reported include a lung cancer at age 45, and three others type unknown. The proband was previously referred to the Medical Genetics program. Correspondence between Alberta

and the Medical Genetics program, regarding the proband's niece who was diagnosed with CRC last year at age 47 is on going.



Pedigree 9 **High age/cancer mod AC -Family 17**

MSI
MSH6-

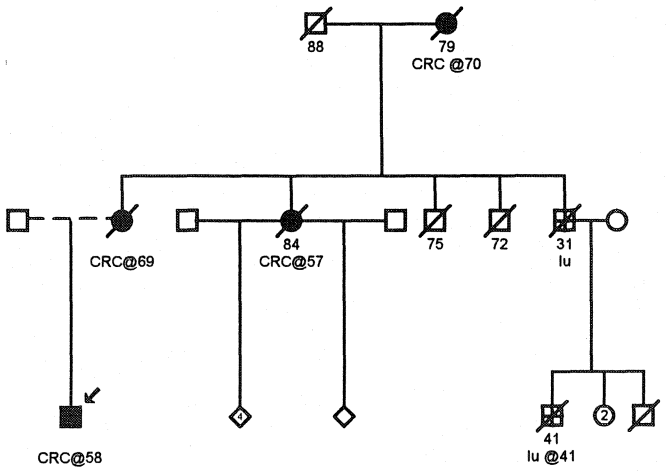
MSI-H and hMLH1- (n=3)

Proband number 14 (pedigree 10) is a 62 year old male who was diagnosed, in 1998, with a moderately differentiated cancer of the cecum at age 58. Two small polyps are reported. A cousin was identified as the proband's proxy because he is mentally delayed. He was born in Point LaHaye, St. Mary's Bay (SMB) and now resides in St. Mary's. His mother and maternal grandparents originated from Point LaHaye, SMB. There is no paternal history available. The pedigree constructed consists of 16 relatives. He has 1 first degree relative, his mother. His mother was diagnosed with a HNPCC cancer, CRC, at age 69. He has 6 second degree relatives. Two HNPCC cancers are reported in his second degree relatives. A maternal aunt was diagnosed with CRC at age 57 and a recurrence at age 84. His maternal grandmother was diagnosed with CRC in her 70's. The proband was previously referred to the Medical Genetics program.

Proband number 50 (pedigree 11) is now deceased. She was diagnosed, in 1997, with a poorly differentiated cancer of the cecum at age 64. Her daughter was identified as her proxy. She resided in Dunville, Placentia Bay. Her father originated from Isle Valen, Placentia, and her mother originated from Dunville, Placentia Bay. The pedigree consists of 56 relatives. She has 14 first degree relatives: 5 children, 7 siblings, and parents. Five HNPCC cancers were diagnosed in first degree relatives. Her mother was diagnosed with a CRC at age 69 and died at age 72. Her father was diagnosed with a CRC at age 62 and died that year. Three siblings were diagnosed with colon cancer; two sisters at ages 51 and 64, and a brother at age 53. No other cancers were reported in first degree relatives but two other siblings had polyps. A daughter had a tubular adenoma removed at age 41 and two other daughters had polyps removed. The proband has 42

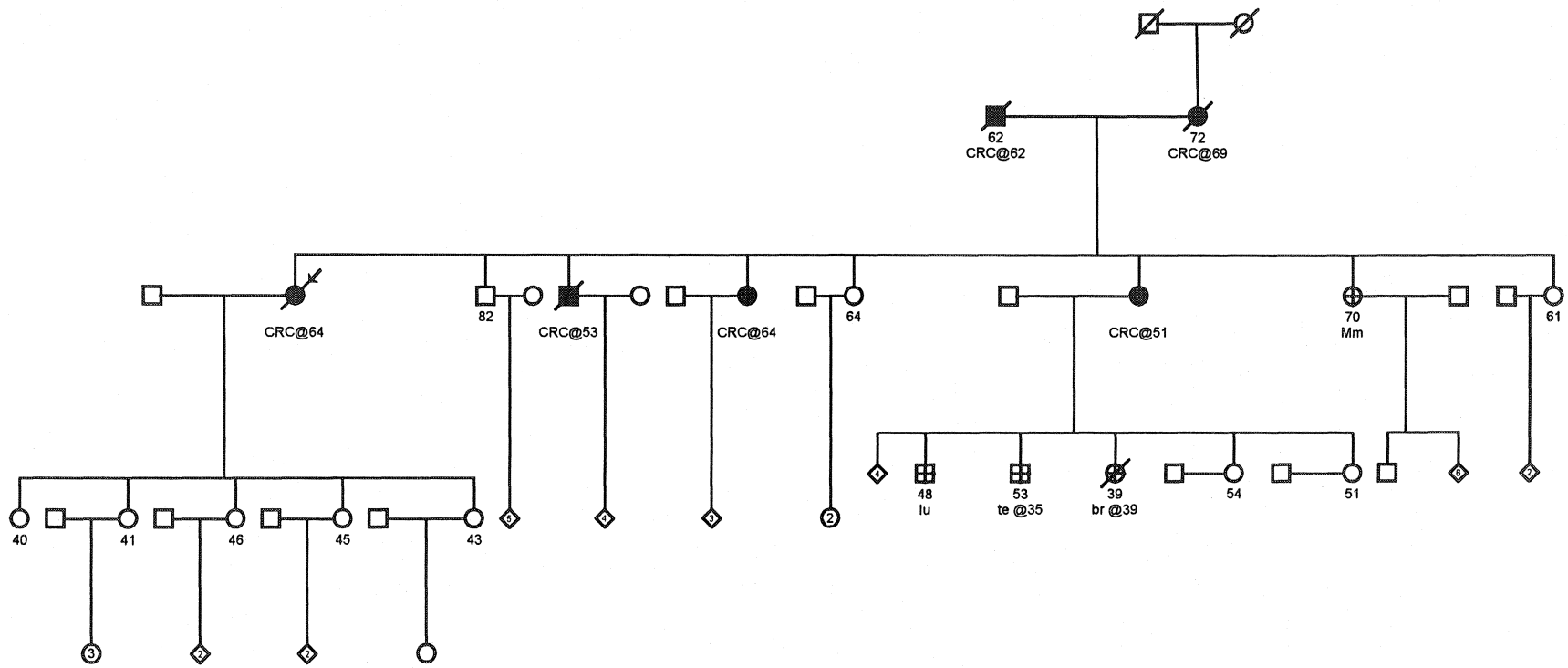
second degree relatives. A nephew was diagnosed with a testicular cancer at age 35 and a niece was diagnosed with a breast cancer at age 39. No other cancers were reported in her immediate family. The proband was previously referred to the Medical Genetics program.

Proband number 78 (pedigree 12) is a 56 year old female who was diagnosed, in 1997, with a poorly differentiated cancer of the ascending colon at age 51. Pathology revealed the presence of 2 sessile polyps and a pedunculated tubulovillous adenomatous polyp. She was born in Tacks Beach, Placentia Bay, and now resides in Renews. Both of her parents and grandparents originated from Tacks Beach, Placentia Bay. The pedigree constructed consists of 101 relatives. She has 19 first degree relatives: 6 children, 11 siblings, and parents. She has 42 second degree relatives. Her father died at age 72 with a HNPCC cancer, stomach cancer. Her mother died of Parkinsons at age 83. A sibling died at 3 months. She has 42 second degree relatives. Three HNPCC cancers are reported in second degree relatives. A paternal uncle was diagnosed with a stomach cancer in his 60's and paternal aunt was diagnosed with an ovarian cancer at age 70. A maternal uncle was diagnosed with a brain cancer in his 70's. A nephew was diagnosed with a leg cancer at age 15. No other cancers were reported in second degree relatives. A HNPCC cancer, a lymphoma and an unknown cancer are reported in paternal cousins. No other cancers were reported in her family.



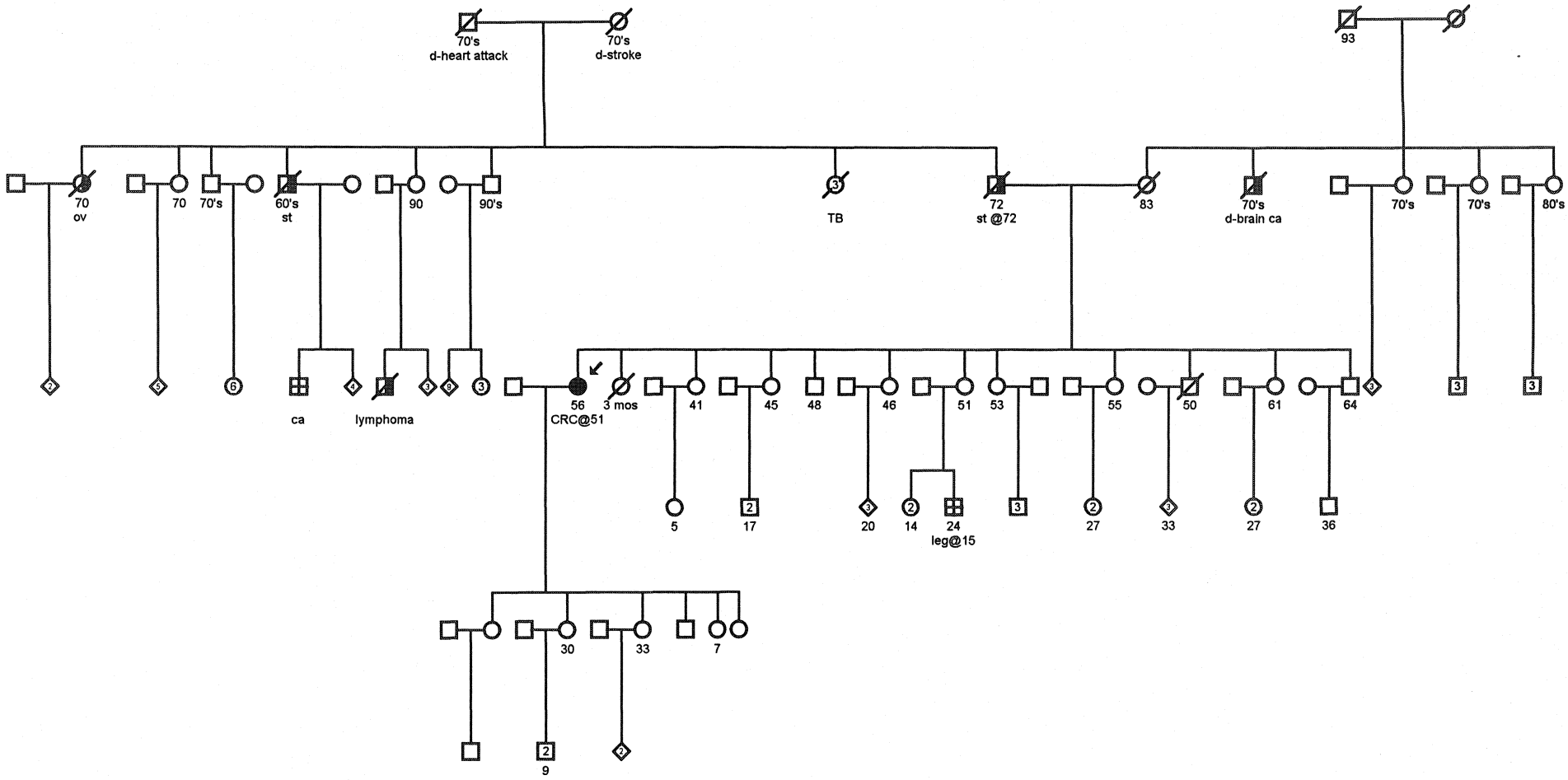
Pedigree 10 High age/cancer mod AC-Family 14

MSI
MLH1-



Pedigree 11 High age/cancer mod AC-Family 50

MSI
MLH1-



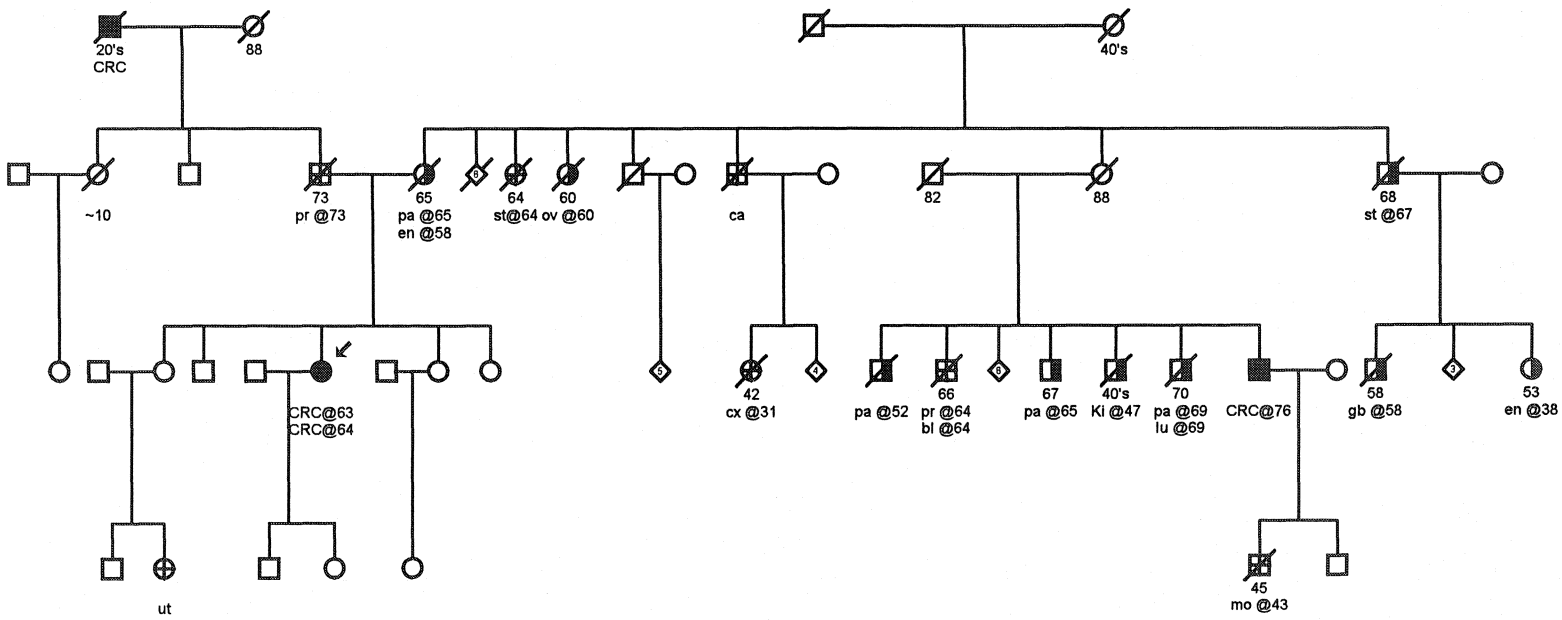
Pedigree 12 High age/cancer mod AC- Family 78

MSI
MLH1-

MSS and no loss (n=10)

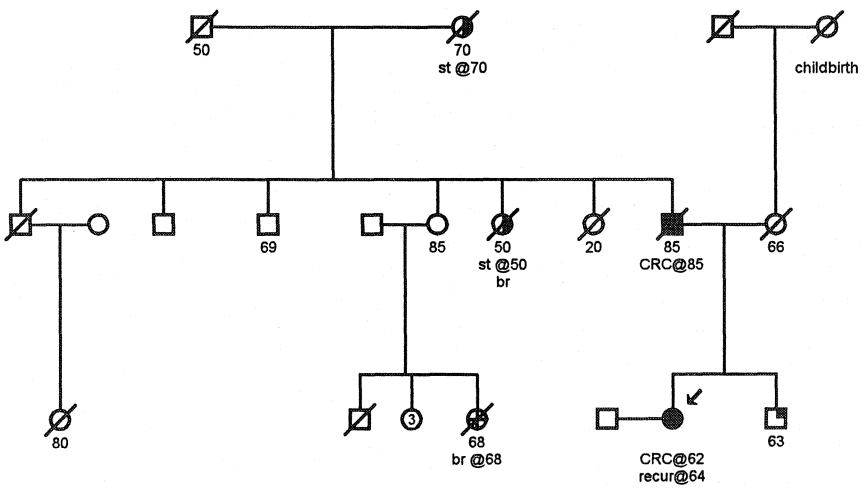
Proband 183 (pedigree 13) is a female who was diagnosed, in 1997, with a moderately differentiated cancer of the sigmoid colon at age 63. In 1998 she was diagnosed with a second primary, a well differentiated cancer of the sigmoid colon. She resides in St. John's. Her parents and maternal grandparents originated from Spaniard's Bay. The pedigree constructed consists of 48 relatives. She has seven first degree relatives: a child, 4 siblings, and parents. Her son was diagnosed with villo-tubular and tubular adenomas at age 39. Her mother was diagnosed with two HNPCC cancers, endometrial cancer at age 58 and pancreatic cancer at age 65. No other cancers were reported in first degree relatives. She has 18 second degree relatives. Four HNPCC cancers are reported in second degree relatives. A maternal uncle was diagnosed with a stomach cancer at age 64. A maternal aunt was diagnosed with a stomach/throat cancer at age 64. A maternal aunt was diagnosed with an ovarian cancer at age 60. Her paternal grandfather is suggested to have died in his 20s of a rectal cancer. No other cancers were reported in second degree relatives. Seven HNPCC cancers are reported in maternal cousins. A maternal cousin was diagnosed with a CRC at age 76. Another maternal cousin was diagnosed with an endometrial cancer at age 38. Gallbladder cancer was diagnosed in a maternal cousin at age 58. Pancreatic cancer was diagnosed in 3 maternal cousins, at ages 52, 65, and 69. A maternal cousin was diagnosed with a kidney cancer (RCC) at age 47. Another maternal cousin was diagnosed with a bladder cancer at age 64 and prostate cancer at age 64. The proband was screened and was negative for the Family C mutation. The proband was previously referred to the Medical Genetics program.

Proband number 57 (pedigree 14) is 66 year old female who was diagnosed, in 1998, with a moderately differentiated cancer of the sigmoid colon at age 62. She was diagnosed with a recurrence in 2000 and another recurrence with liver metastases in 2001. Previous history reveals a hysterectomy and bilateral salpingo-oophorectomy at 31. She was born in Bar Haven, Placentia Bay and now resides in St. John's. Her mother and her maternal grandfather originated from Great Paradise, Placentia Bay. The origin of her maternal grandmother was not given. Her father and her paternal grandparents originated from Bar Haven, Placentia Bay. The pedigree constructed consists of 19 relatives. She has three first degree relatives: 1 sibling, and parents. Her father was diagnosed with a HNPCC cancer, CRC, at age 86. She has 10 second degree relatives. A paternal aunt was diagnosed with a HNPCC cancer, stomach cancer, at age 50. No other cancers were reported in this family.



Pedigree 13 High age/cancer mod AC-Family 183

MSS
no loss



Pedigree 14 High age/cancer mod AC-Family 57

MSS
no loss

Proband number 70 (pedigree 15) is a 68 year old man who was diagnosed, in 1997, with a poorly differentiated cancer of the sigmoid colon at age 63. Chart review indicated the presence of polyps: in 1989 an adenomatous polyp and 5 hyperplastic polyps, in 2000 a tubular polyp and a cancer insitu, and in 2002 two polyps were reported. He was born in St. John's where he now resides. His mother and maternal grandparents originated from Bay L'Argent, Fortune Bay. His father and paternal grandfather originated from St. John's. His paternal grandmother originated from Manuels. The pedigree constructed consists of 77 relatives. He has ten first degree relatives: 2 children, 6 siblings, and parents. His mother died at age 98 and his father died at age 97. No other cancers were reported in first degree relatives. He has 32 second degree relatives. A maternal aunt was diagnosed with a stomach cancer younger than age 80. His maternal grandfather was diagnosed with an ear cancer and died at age 85. His paternal grandmother was diagnosed with a stomach cancer at age 85. No other cancers were reported second degree relatives. Four HNPCC cancers are reported in maternal cousins. They include CRC diagnosed at ages 46, 59, and 66 and a pancreatic cancer diagnosed at age 58. A paternal cousin was diagnosed with a lung cancer at age 61.

Proband number 39(pedigree 16) is a 65 year old male who was diagnosed, in 1997, with a moderately differentiated cancer of the rectum at age 60. Pathology revealed the presence of a hyperplastic polyp at time of surgery. A tubular polyp with moderate dysplasia is reported on follow-up colonoscopy in 1998. He was born in Stones Cove, Fortune Bay and now resides in St. John's. His mother and his maternal grandfather originated from Stones Cove, Fortune Bay. His maternal grandmother originated from Belloram, Fortune Bay. His father originated from St. Jacques, Fortune Bay. His

paternal grandmother originated from English Harbour, Fortune Bay. His paternal grandfather originated from Dublin, Ireland. The pedigree constructed consists of 54 relatives. He had fourteen first degree relatives: 5 children, 7 siblings, and parents. Two HNPCC cancers are reported in first degree relatives. His father died at age 89 from a stomach cancer. A brother was diagnosed with a pancreatic cancer at age 61. No other cancers were reported in his first degree relatives. His mother died at 79 from heart disease. He had 33 second degree relatives. A HNPCC cancer, stomach cancer, is reported in a maternal uncle at approximately age 50. No other cancers were reported in the family.

Proband number 99 (pedigree 17) is a 73 year old female who was diagnosed, in 1997, with a moderately differentiated cancer of the splenic flexure at age 68. Chart review indicates a history of a polyp removed in 1985 which had adenomatous changes and a possible adenocarcinoma at the top of the polyp. Another polyp in 1997 was also revealed to be a small tubular adenoma. She was born in Bay Roberts. Her mother originated from Holyrood. Her maternal grandparents originated form Salmonier. Her father and paternal grandfather originated from Job's Cove, Carbonear. The origin of her paternal grandmother was not given. The pedigree constructed consisted of 57 relatives. She had 12 first degree relatives; 5 children, 5 siblings, and parents. A HNPCC cancer, CRC, is reported in her mother at age 60. A sister was diagnosed with uterine cancer at age 47. There are no other reported cancers in first degree relatives. Her father and three siblings are deceased from unrelated causes. She has 25 second degree relatives. A maternal aunt was diagnosed with a HNPCC cancer, CRC, at age 75. No other cancers were reported in second degree relatives. HNPCC cancers, CRC, were reported in two

maternal cousins (siblings) at 59. A maternal cousin was diagnosed with a breast cancer, age unknown. A paternal cousin was diagnosed with a HNPCC cancer, lymphoma at age 50. No other cancers were reported in this family.

Proband number 109 (pedigree 18) is a 59 year old man who was diagnosed, in 1998, with a poorly differentiated cancer of the rectum at age 55. Chemotherapy and radiation were required. He was born in Newtown, Bonavista Bay and now resides in St. John's. His mother and maternal grandfather originated form Cape Island, Bonavista Bay. His maternal grandmother originated from Cape Freels. His father and paternal grandparents originated from Newtown. The pedigree constructed consists of 41 relatives. He has 6 first degree relatives: 3 children, 1 sibling, and parents. His mother was diagnosed with a HNPCC cancer, CRC, at age 72. His father was diagnosed with prostate cancer at age 76 and died at age 79. He has 17 second degree relatives. A maternal uncle was diagnosed with a HNPCC cancer, brain cancer, at age 62. His paternal grandfather was diagnosed with prostate cancer. No other cancers were reported in the family.

Proband number 141 (pedigree 19) is a 63 year old male who was diagnosed, in 1998, with a moderately differentiated cancer of the ascending colon at age 58. He was diagnosed, in 1999, with a malignant melanoma. He was born in Garnish and resided in Chapel's Cove. His mother and maternal grandparents originated from Garnish. His father and paternal grandparents originated from Moreton's Harbour. The pedigree constructed consists of 59 relatives. He has 14 first degree relatives: 4 children, 8 siblings, and parents. His mother was diagnosed with a HNPCC cancer, small bowel cancer, at age 54 and died at age 55. His father died at age 61 from diabetes

complications. His brother was diagnosed with prostate cancer at age 73. No other cancers were reported in his first degree relatives. Two HNPCC cancers were reported in second degree relatives. His maternal grandfather was diagnosed with CRC at age 81. A maternal uncle was diagnosed with a CRC at age 60. He has 41 second degree relatives. No other cancers were reported in the family.

Proband number 151(pedigree 20) is a 66 year old male who was diagnosed, in 1997, with a moderately differentiated cancer of the rectosigmoid junction at age 60. Pathology reveals the presence of a polyp. He was born in St. John's where he now resides. His mother originated from St. John's. His maternal grandparents originated from Western Bay. His father and paternal grandfather originated from Harbour Grace. The demographics of his paternal grandmother were not known. The pedigree constructed consists of 33 relatives. He has 8 first degree relatives, 6 siblings and parents. Two HNPCC cancers were reported in first degree relatives. His mother died from CRC at age 94. His sister was diagnosed with CRC at age 64. His father died from heart complications at age 91. He has 25 second degree relatives. No other cancers were reported in the family.

Proband number 163 (pedigree 21) is a 65 year old female who was diagnosed, in 1997, with a well differentiated cancer of the ascending colon at age 60. Chart review revealed the presence of 2 polyps. She was born in St. John's where she now resides. Her parents and grandparents originated from St. John's. The pedigree constructed consisted of 70 relatives. She had 12 first degree relatives: 5 children, 5 siblings, and parents. Her mother was diagnosed (age unknown) with a HNPCC cancer, colorectal cancer, and died at age 73 from a heart attack. Cervical cancer was diagnosed in her

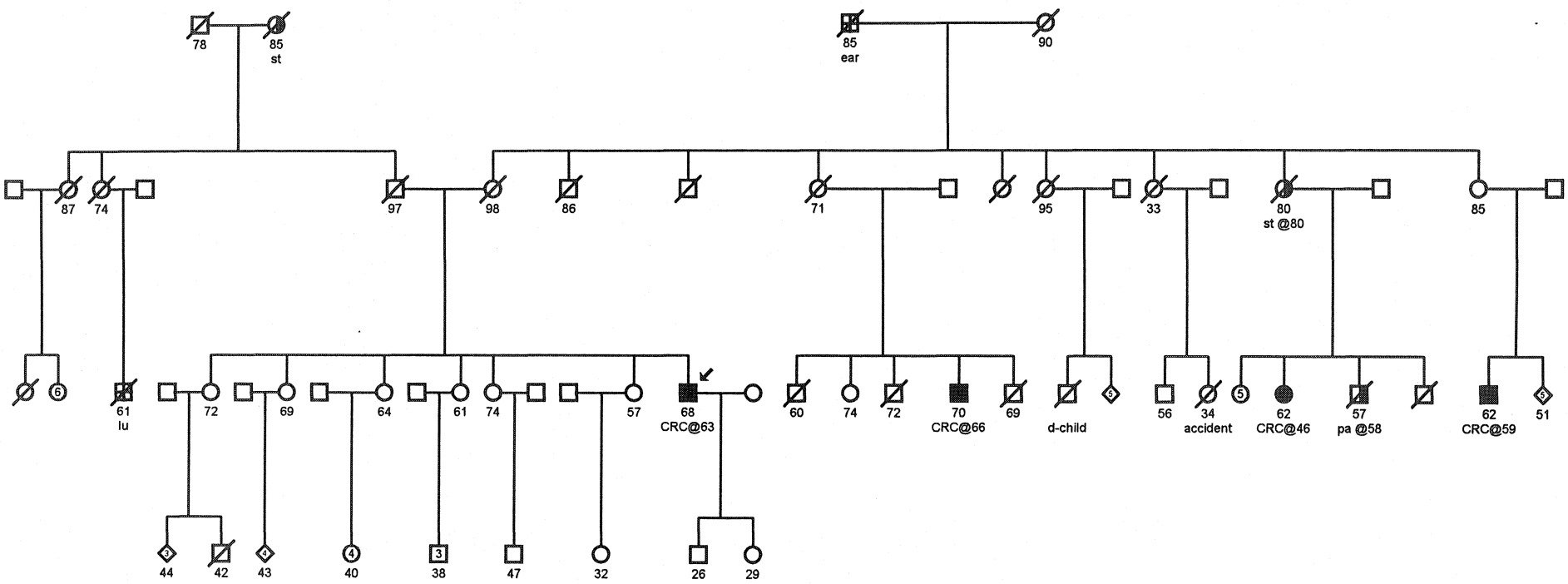
sister at age 67. No other cancers were reported in first degree relatives. A brother is deceased at age 48 due to heart complications. Her father died at age 61 from a heart attack. She has 37 second degree relatives. A HNPCC cancer, CRC, was diagnosed in a maternal aunt at age 63. Two HNPCC cancers were reported in maternal cousins; a CRC at age 53 (cousin is participant in the NFCCR) and an ovarian cancer (age unknown). A maternal cousin died at age 13 due to leukemia. There were no other reported cancers in this family. The proband was previously referred to the Medical Genetic program.

Proband number 189(pedigree 22) is a 56 year old female who was diagnosed, in 1997, with a cancer of the rectum at age 51. She resides in Portugal Cove. Her extended family originated from Portugal Cove. The pedigree constructed consisted of 64 relatives. She has 23 first degree relatives: 21 siblings, and parents. Two HNPCC cancers are reported in first degree relatives. Her mother was diagnosed with a CRC at age 65, a breast cancer at age 68 and died at age 74. Her father was diagnosed with CRC at age 75. A sister was diagnosed with a breast cancer at age 60. She has 11 second degree relatives. Four HNPCC cancers were reported in second degree relatives. Her maternal grandfather was diagnosed with a HNPCC cancer, pancreatic cancer at age 72. Two maternal aunts were diagnosed with double breast cancer primaries at ages 55, 56, and 43 and 72. Cancers reported in maternal cousins include a HNPCC cancer, CRC, at age 45, cervical cancer at age 45, a breast cancer at age 37, and bilateral breast cancers at ages 34 and 54. Basal cell cancer (bcc) at age 40 and breast cancers at ages 41 and 42 were reported in another cousin. This proband is a member of a previously identified hereditary breast cancer family. Extension of her maternal grandfather's family identifies

a strong family history of breast cancer. There is no hereditary breast cancer mutation known for this family.

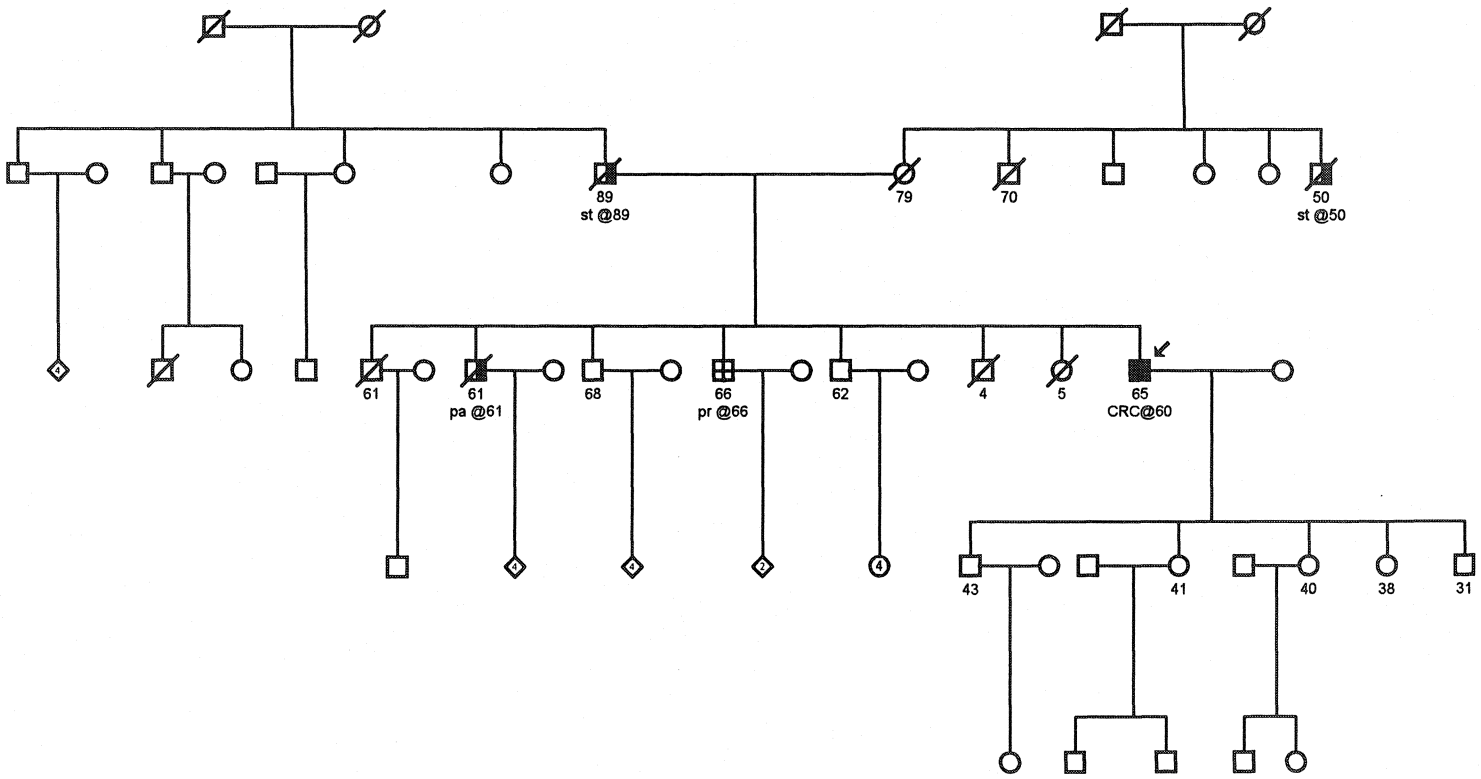
Pending Laboratory Analysis (n=1)

Proband number 104 (pedigree 23) is a 74 year old female who was diagnosed, in 1997, with a moderately differentiated cancer of the descending colon at age 69. She has a history of crohns/colitis. She was born in St. John's where she now resides. Her mother and her maternal grandmother originated from Kingscove, Bonavista Bay. Her maternal grandfather originated from Isle au Bois, Labrador. Her father and her paternal grandparents originated from St. John's. The pedigree constructed consists of 69 relatives. She has 13 first degree relatives: 2 children, 9 siblings, and parents. Three HNPCC cancers were reported in first degree relatives. A CRC was reported in a brother at age 57, and oesophageal cancer was reported in another brother at age 51, and a stomach cancer was reported in his father at age 86. A lung cancer was reported in a brother at age 53. She has 52 second degree relatives. A HNPCC cancer, CRC, was reported in a nephew at age 51. A niece died at age 38 due to liver metastases from an ocular melanoma. There were no other reported cancers in second degree relatives. A HNPCC cancer, CRC, was reported in a paternal cousin. An unknown cancer was reported in another paternal cousin. There were no other reported cancers in this family. The proband was previously referred to Medical Genetics program.



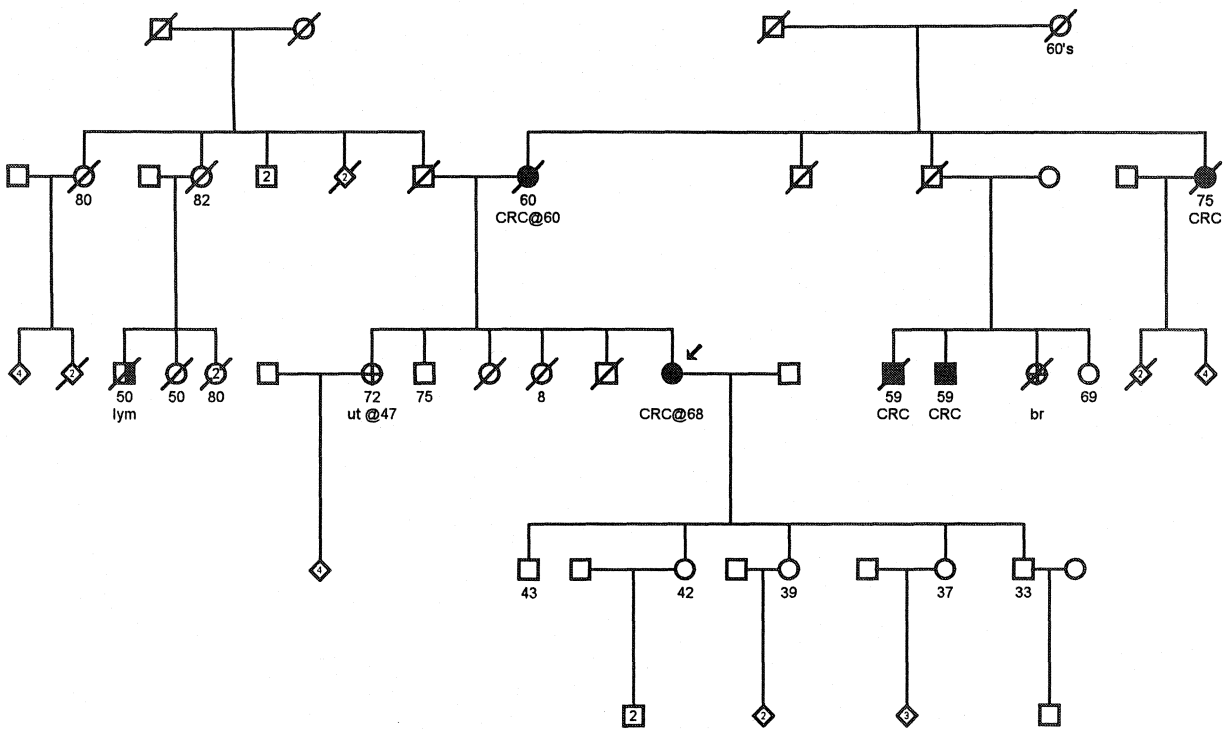
Pedigree 15 High age/cancer mod AC- Family 70

MSS
no loss



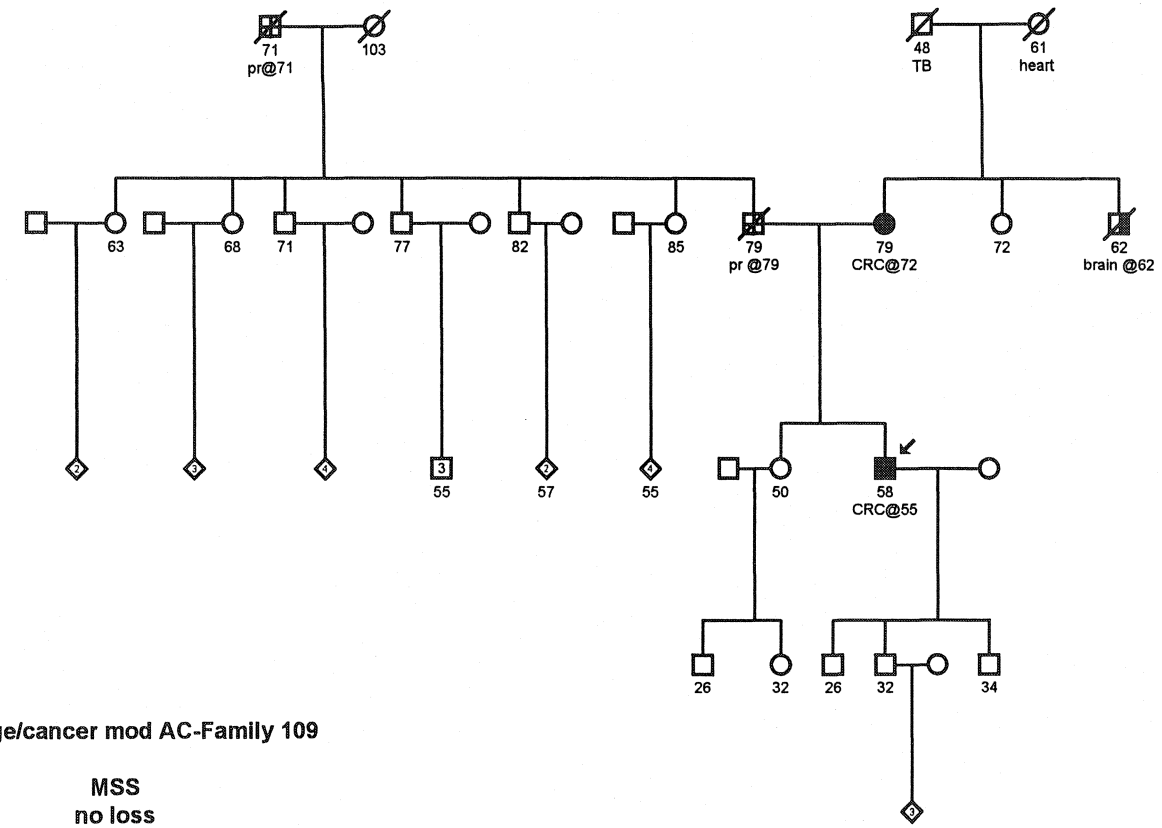
Pedigree 16 High age/cancer mod AC-Family 39

MSS
no loss



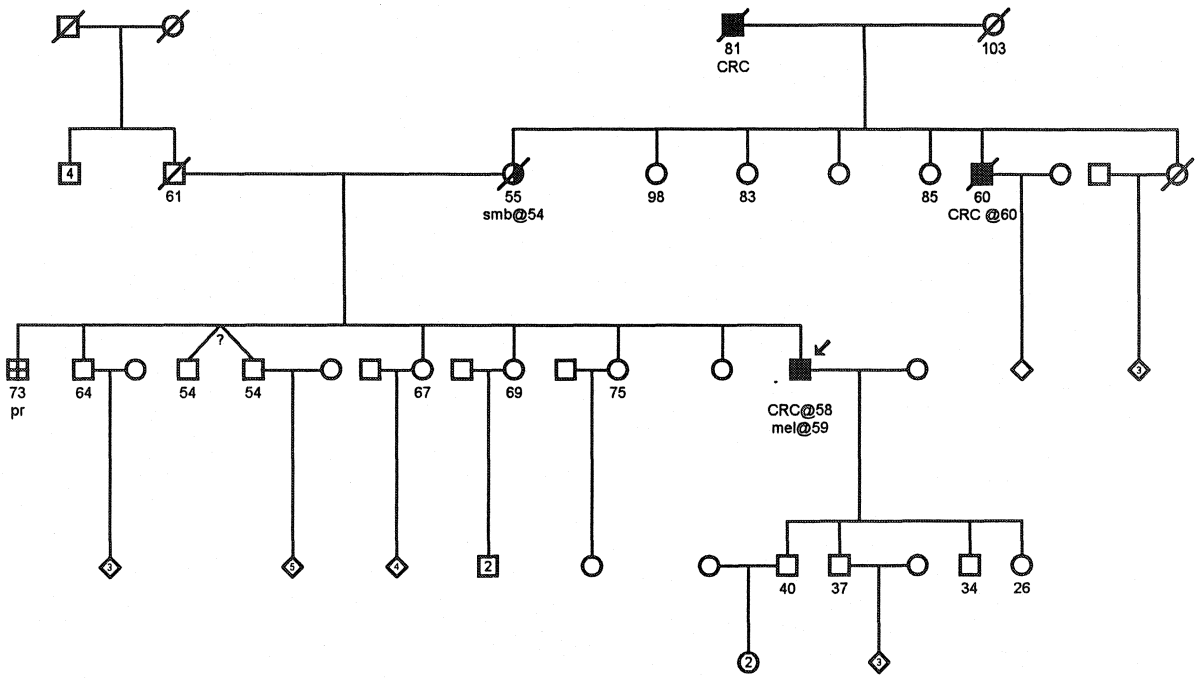
Pedigree 17 **High age/cancer mod AC-Family 99**

MSS
no loss



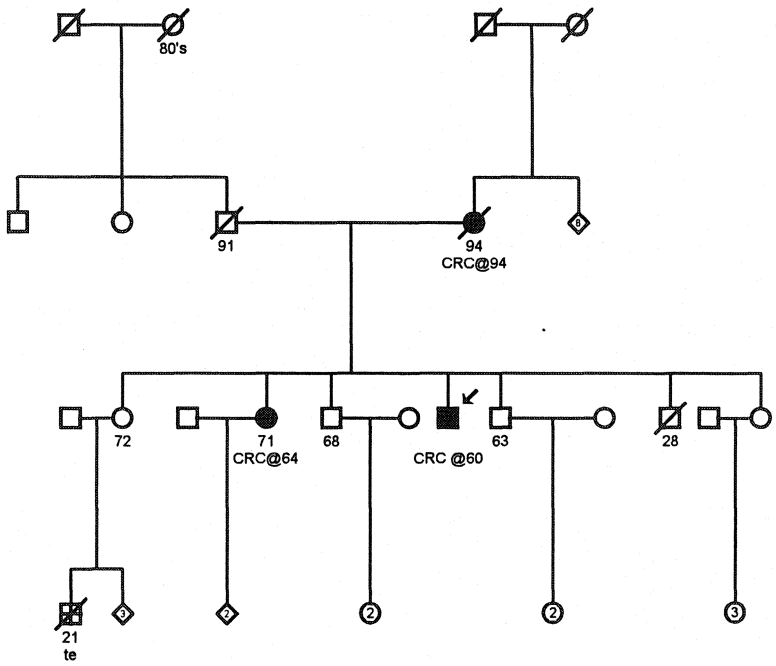
Pedigree 18 **High age/cancer mod AC-Family 109**

MSS
no loss



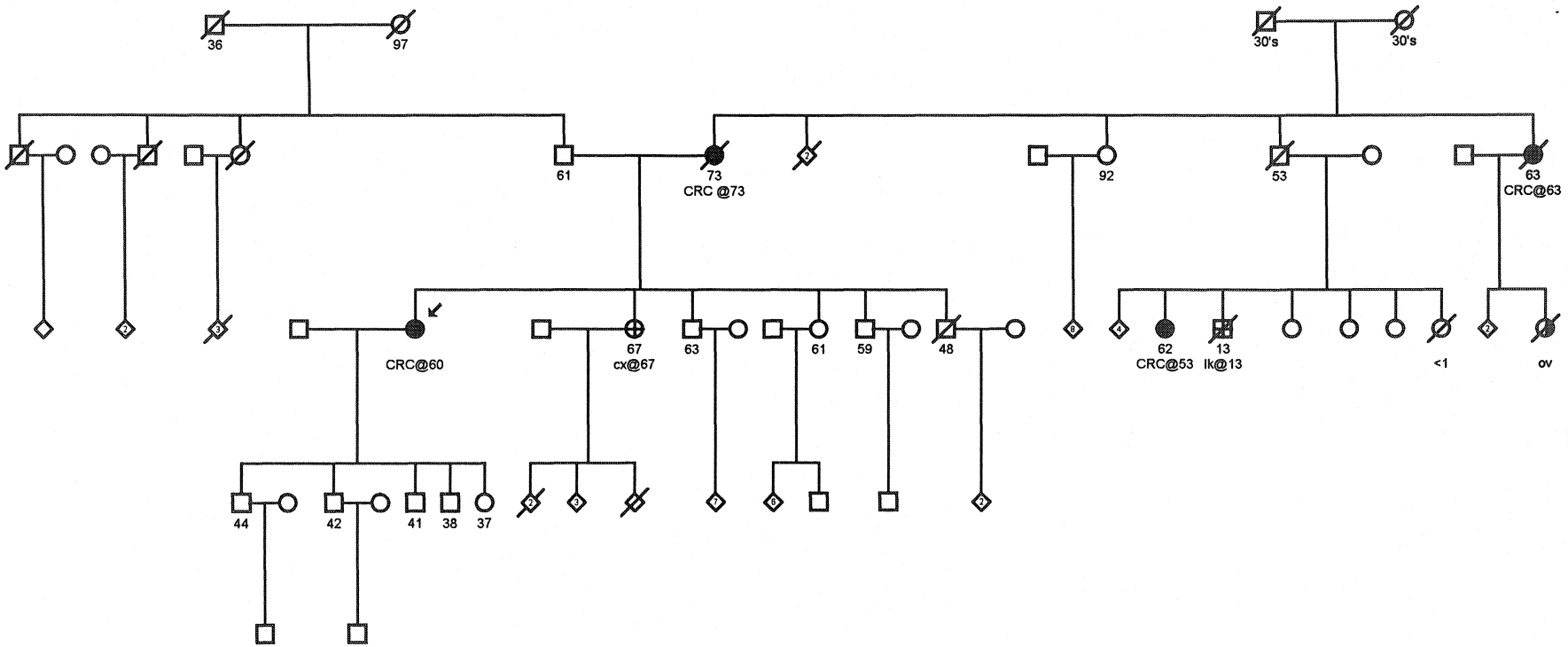
Pedigree 19 **High age/cancer mod AC-Family 141**

MSS
no loss



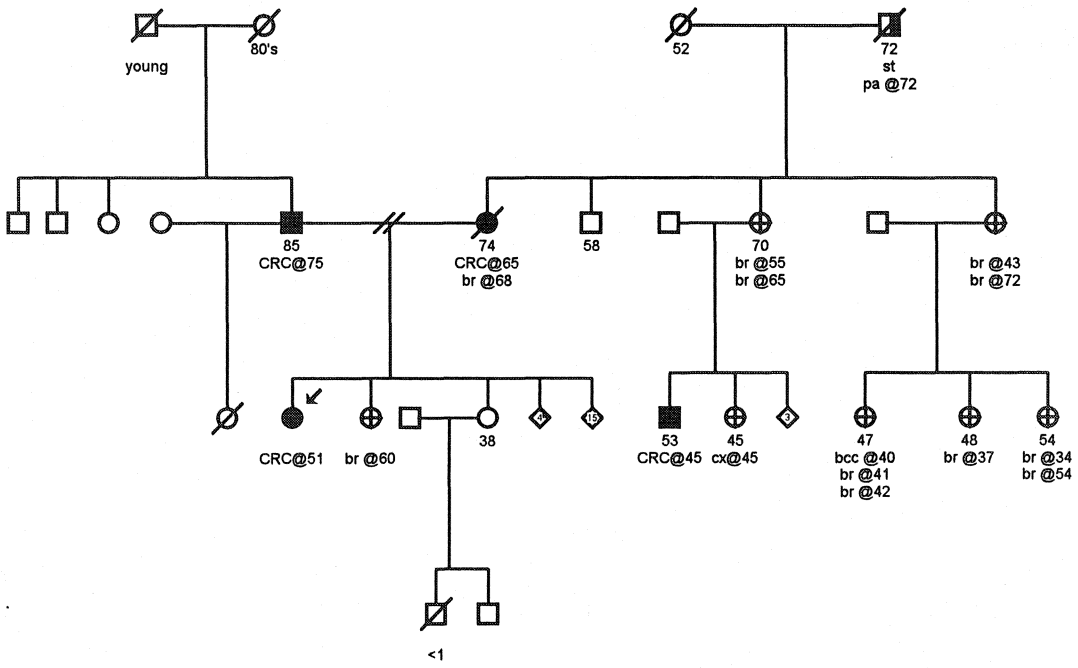
Pedigree 20 High age/cancer mod AC-Family 151

MSS
no loss



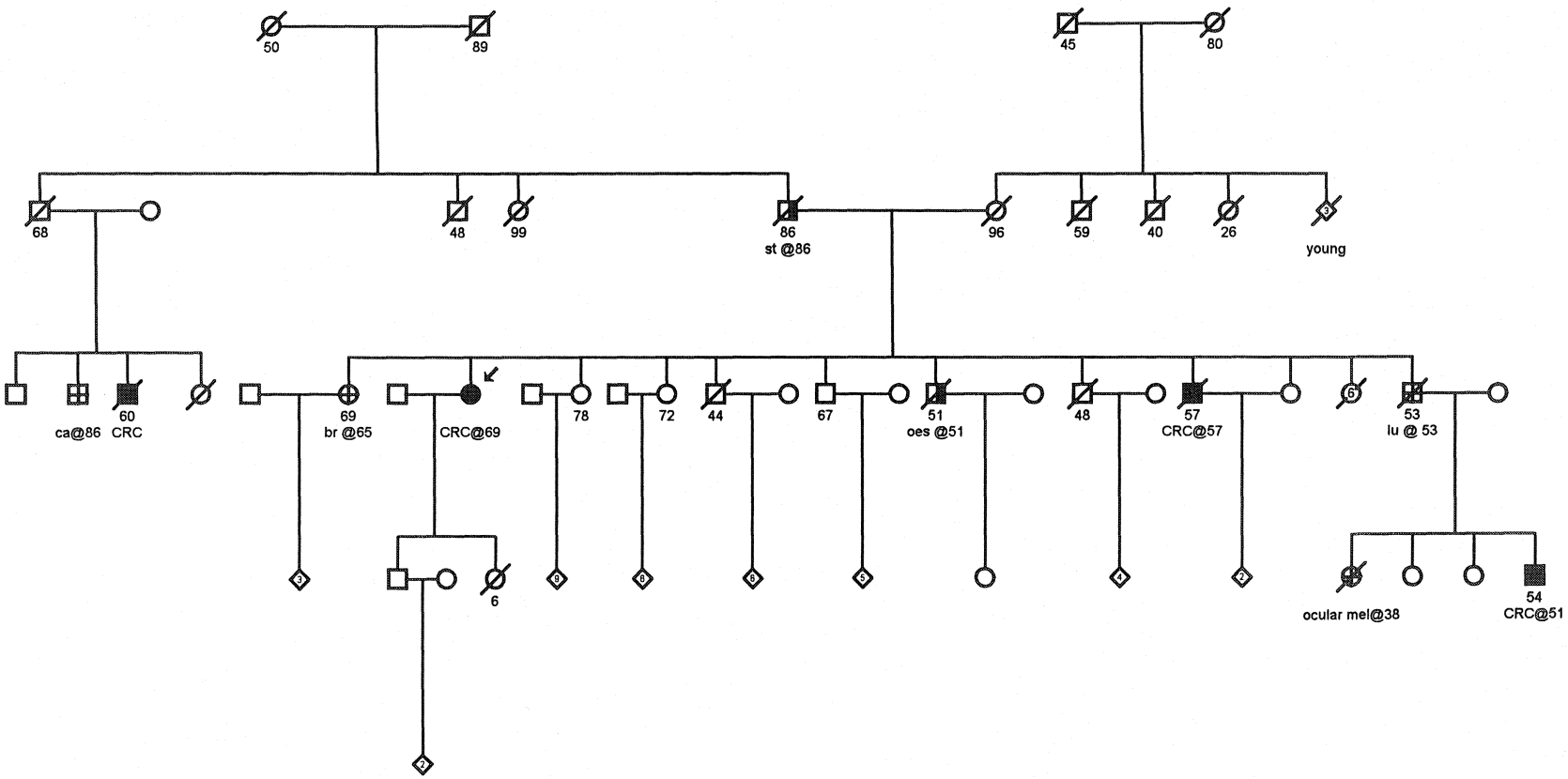
Pedigree 21 High age/cancer mod AC-Family 163

MSS
no loss



Pedigree 22 High age/cancer mod AC-Family 189

MSS
no loss



Pedigree 23 **High age/cancer mod AC-Family 104**

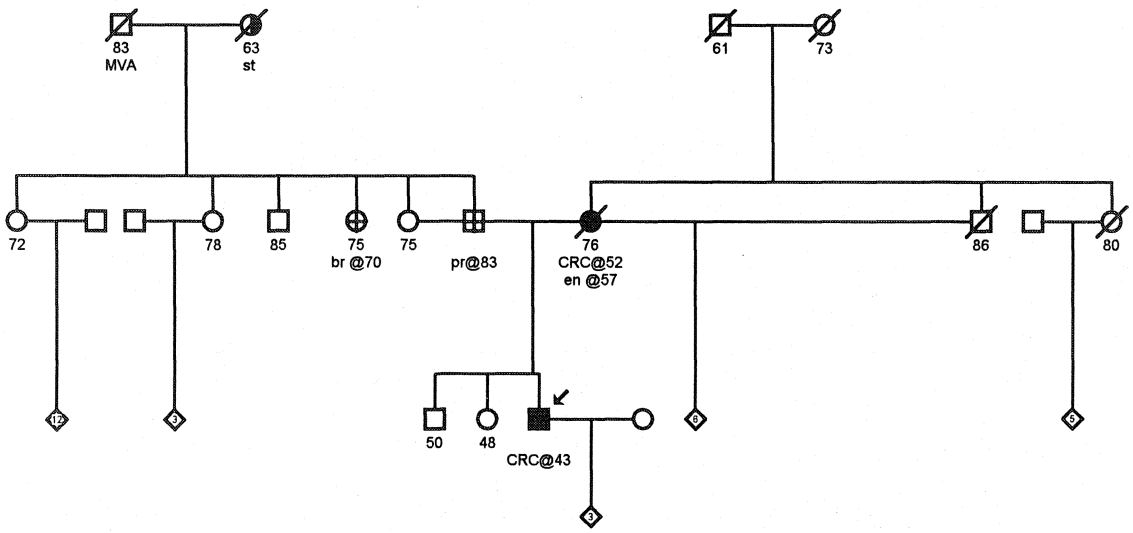
Pending Laboratory

3.72 INTERMEDIATE RISK FAMILIES (n= 22 families)

INTERMEDIATE Criteria #1 (n= 14)

MSI-H, hMSH2- and hMSH6- (n= 1 family)

Proband number 45 (pedigree 24) is a 48 year old male who was diagnosed, in 1997, with a moderately differentiated cancer of the cecum at age 43. Pathology revealed the presence of two polyps and chart review indicated the presence of two small hyperplastic polyps in 1999 and 4 hyperplastic polyps in 2000. He now resides in Paradise. His maternal grandmother originated from Fox Cove and his maternal grandfather originated from Oderin, PB. His paternal grandmother originated from Little Paradise and his paternal grandfather originated from Little Bonavista. The pedigree constructed consists of 35 relatives. He has 7 first degree relatives; 3 children, 2 siblings, and parents. He has 11 second degree relatives. Two HNPCC cancers were reported in his mother. She was diagnosed with a CRC at age 52, an endometrial cancer at age 57, a villous adenoma at age 60, a villous adenoma at age 71, and a tubular adenoma at age 74. She died at age 76. His father was diagnosed with prostate cancer. Other cancers reported include a HNPCC cancer, stomach cancer, in his paternal grandmother at age 63 and a breast cancer in a paternal aunt at age 70. This proband was previously referred to the Medical Genetics program.



Pedigree 24 Intermediate 1-Family 45

MSI
MSH2- and MSH6-

MSI-H and hMLH1- (n = 1 family)

Proband number 162 (pedigree 25) is a 72 year old female who was diagnosed, in 1998, with a moderately differentiated cancer of the cecum at age 68. Pathology indicates the presence of numerous polyps. She was born in Port de Grave and her mother and maternal grandfather originated from Bishop's Cove. Her maternal grandmother originated from Blaketown. Her father and paternal grandparents originated from Port de Grave. The pedigree constructed consists of 66 relatives. She has 12 first degree relatives; 4 children, 6 siblings, and parents. Her brother was diagnosed with a HNPCC cancer, CRC, at age 64. No other cancers were reported in first degree relatives. Three siblings have had polyps removed. Her father died at age 92 and her mother died at age 83 from heart complications. A sibling is deceased at age 32 due to an accident. She has 34 second degree relatives. A HNPCC cancer, CRC, was diagnosed in a maternal aunt at age 70. A stomach cancer was reported in a maternal cousin at age 69, and breast cancer was reported in another maternal cousin. Unknown cancers were reported in her maternal grandmother, a maternal uncle, and a maternal cousin. A review of the pedigree connected this family through marriage to proband #100 in this study.

MSI-H and pending IHC Analysis (n = 1 family)

Proband number 10 (pedigree 26) is now deceased. He was diagnosed, in 1997, with a cancer of the rectosigmoid junction at age 65. He died three months following his diagnosis. He was previously diagnosed with a cancer of the colon in 1982. He underwent a right hemicolectomy at that time. He resided in St. John's. His mother and maternal grandmother originated from Islington, Trinity Bay. The origin of his maternal grandfather was not given. His father and his paternal grandfather originated from

Bishop's Cove. His paternal grandmother originated from Victoria, Carbonear. The pedigree constructed consists of 41 relatives. He had 12 first degree relatives: 4 children, 6 siblings, and parents. Two HNPCC cancers, CRC, were diagnosed in siblings; at age 60 in a sister, and at age 69 in a brother. His father died at age 55 from heart complications and his mother died at age 74 form diabetes complications. Two of the proband's brothers were diagnosed with colitis. No other cancers were reported in first degree relatives. He has 27 second degree relatives. A paternal uncle was diagnosed with a lung cancer at a young age. A paternal cousin was diagnosed with a breast cancer at age 56. No other cancers were reported in the family.

MSS and no loss (n = 8 families)

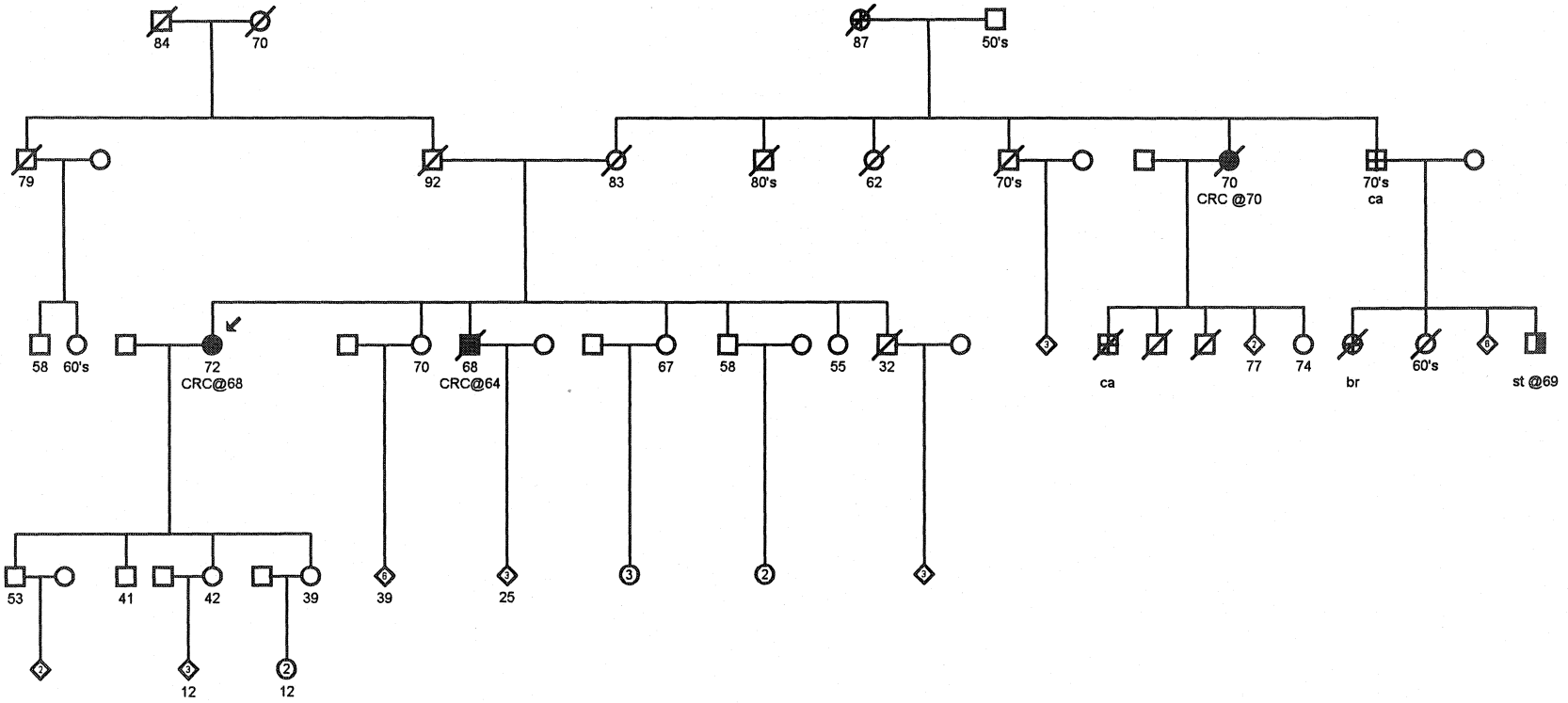
See appendix A, pedigrees 34 through 41

MSS and pending IHC (n = 2 families)

See appendix A, pedigrees 42 through 43

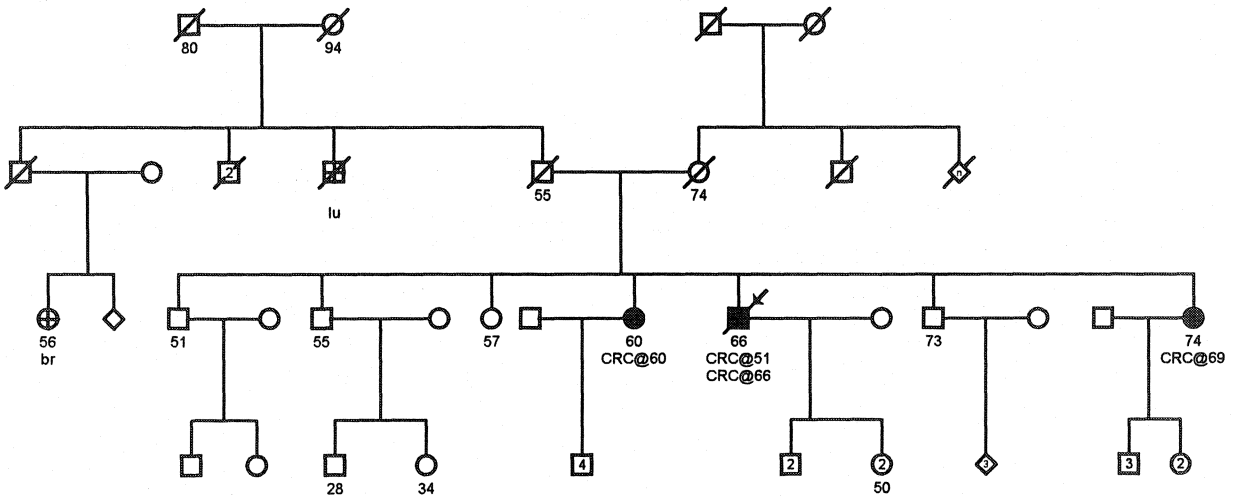
No sample available for molecular and pathology analysis (n =1 family)

See appendix A, pedigree 44



Pedigree 25 Intermediate 1- Family 162

MSI
MLH1-



Pedigree 26 **Intermediate 1-Family 10**

MSI
pending IHC

INTERMEDIATE RISK 2 (proband and relative with CRC ≤35) (n = 1 family)

MSI-H and hMSH2- and hMSH6-

Proband number 135 (pedigree 27) is a 56 year old male who was diagnosed, in 1997, with a well differentiated cancer of the cecum at age 51. Pathology revealed the presence of three polyps and one was indicated to be a tubular adenoma. Follow-up colonoscopy revealed a tubular adenoma in 1999. He was born in Charleston, Bonavista Bay. His Mother and maternal grandparents originated from Cannings Cove, Bonavista Bay. His father and his paternal grandparents originated from Charleston, Bonavista Bay. The pedigree constructed consists of 77 relatives. He has 6 first degree relatives; 4 sibling, and parents. Three siblings are deceased at ages 30, 45, and 50 from unrelated causes. His mother died at 54 from heart complications. He also reported that his mother had a questionable cancer, type unknown. There were no other cancers reported in first degree relatives. His father died of 78 after a stroke. His sister had had colonoscopy screening. He has 26 second degree relatives. Two HNPCC cancers were reported. A CRC was diagnosed in a maternal uncle at age 60 and a CRC is reported in a maternal cousin at age 35. No other cancers were reported in the family.

INTERMEDIATE RISK 5- (DOUBLE PRIMARIES) (n = 3 families)

MSI-H and hMLH1- (n=1 family)

Proband number 152 (pedigree 28) is a 62 year old female who was diagnosed, in 1998, with 2 poorly differentiated cancers of the colon at age 56. She was born in St. John's where she now resides. Her parents and her grandparents originated form St. John's. The pedigree constructed consists of 77 relatives. She has 15 first degree relatives: 6 children, 7 siblings, and parents. Her mother died of heart complications at

age 76. Her father also died of heart complications at age 68. Her son had a skin cancer at age 34. A brother died from heart complications at age 38, another brother died accidentally at age 27. A sister died from lung cancer with metastasis at age 59. No other cancers were reported in her first degree relatives. She has 49 second degree relatives. A niece was diagnosed with a HNPCC cancer, brain cancer, at age 27. A maternal cousin was diagnosed with lung cancer with metastasis at age 65. No other cancers were reported in the family.

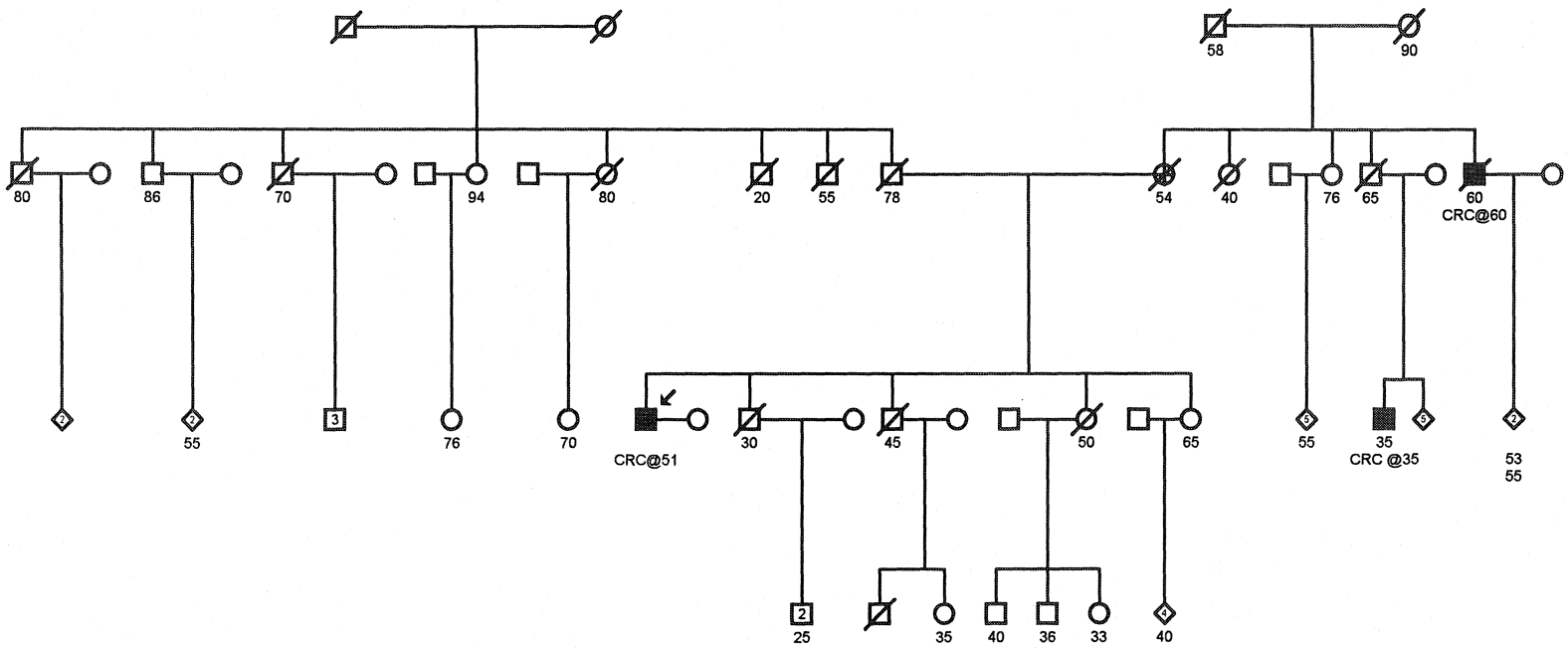
MSS and no loss (n= 2)

See appendix A, pedigrees 45 through 46

INTERMEDIATE RISK 7- (n = 4 families)

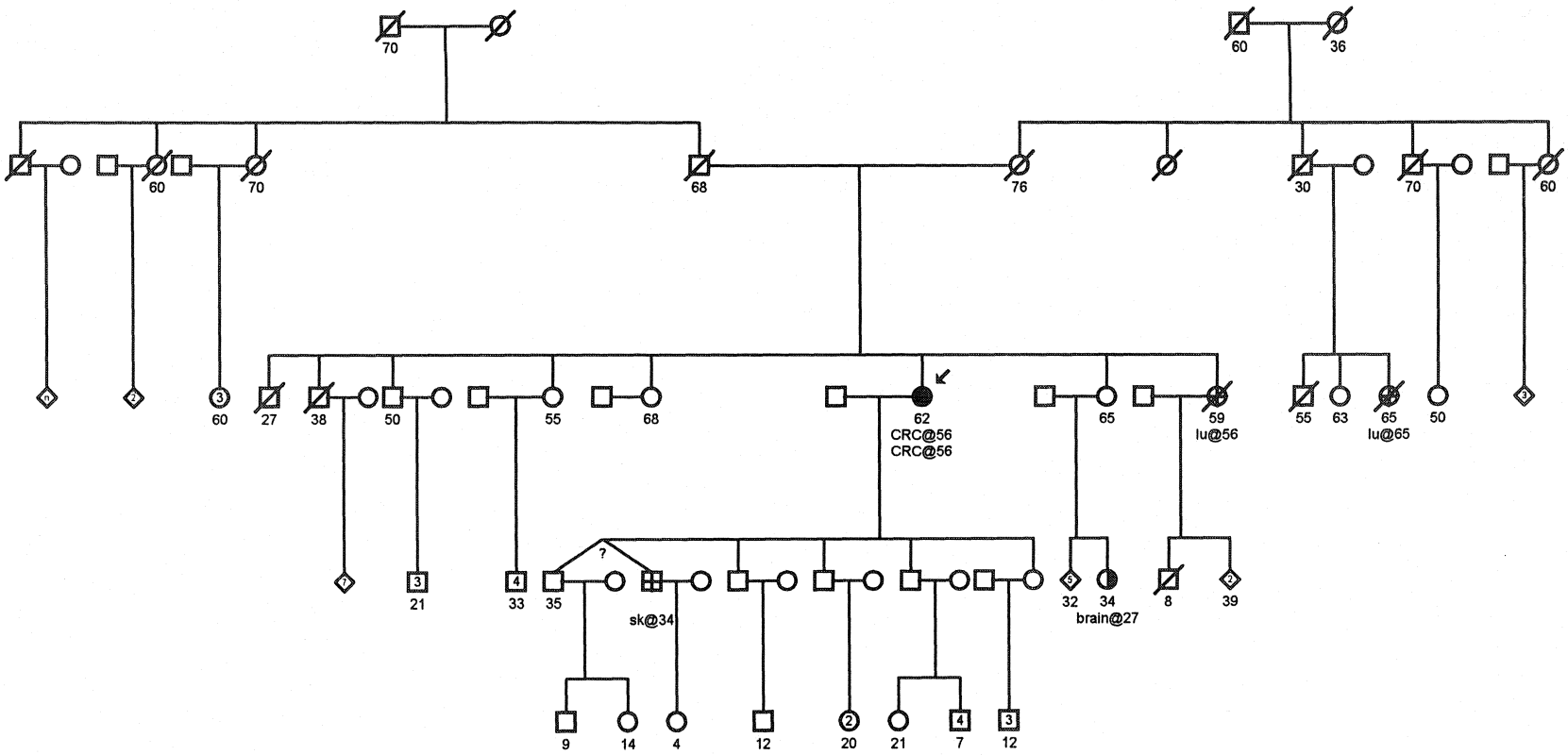
MSS and no loss (n=4 families)

See appendix A, pedigrees 47 through 50



Pedigree 27 **Intermediate 2-Family 135**

MSI
MSH2- and MSH6-



Pedigree 28 Intermediate 5-Family 152

MSI
MLH1-

3.73 LOW RISK FAMILIES (n = 34 families)

INFORMATIVE FAMILIES (n= 21)

MSI-H and hMLH1- (n=1)

Proband number 108 (pedigree 29) is a 60 year old male who was diagnosed, in 1998, with a moderately differentiated cancer of the cecum at age 55. Chart review indicated the removal of polyps in follow-up colonoscopies. He was born in Freshwater, Carbonear where he now resides. His mother and maternal grandfather originated from Freshwater, and his maternal grandmother originated from Victoria. His father and paternal grandfather originated from Freshwater, and his paternal grandmother originated from Bristol's Hope. The pedigree constructed consists of 60 relatives. He has 8 first degree relatives: 3 children, 3 siblings, and parents. His mother died of heart complications at 78. His father is still alive and is 82 years old. Polyps have been reported in a sibling who is 54 years old. No other cancers were reported in his first degree relatives. He has 25 second degree relatives. There are 2 breast cancers reported in a paternal aunt at age 65 and 70. No other cancers were reported in the family.

MSI-H and hMSH6- (n= 1)

Proband number 180 (pedigree 30) is a 43 year old female who was diagnosed, in 1998, with a poorly differentiated cancer of the cecum at age 38, and also a breast cancer at 38. She emigrated from Libya but now resides in St. John's. The pedigree constructed consists of 23 relatives. She has 10 first degree relatives; 8 siblings, and parents. She has 13 second degree relatives. No other cancers were reported in this family. She was previously referred to Medical Genetics because she was diagnosed with a double primary at a young age.

MSI-H and no loss (n= 1)

Proband number 63 (pedigree 31) is a 74 year old male who was diagnosed, in 1997, with a moderately differentiated cancer of the cecum at age 68. Pathology reveals the presence of two tubulovillous polyps. He was previously referred to Medical Genetics because of a family history of breast cancer. She was born in Upper Gullies (Kelligrews) and now resides in Bay Roberts. His mother and maternal grandparents originated from Foxtrap. His father and her paternal grandparents originated from Upper Gullies. The pedigree constructed consists of 36 relatives. He has 11 first degree relatives: 3 children, 6 siblings, and parents. His mother died at age 62 due to a cerebral haemorrhage. His father died at age 84 due to emphysema. A sister was diagnosed with a breast cancer and subsequently died at age 64 due to emphysema. Another sister is dead at age 69 due to a heart attack. A sister had pre-cancerous polyps and part of her bowel removed before age 64. A brother had polyps removed. No other cancers were reported in his first degree relatives. He has 11 second degree relatives. A cancer (type unknown) was reported in a maternal uncle. He also reported a breast cancer in her maternal grandmother who died at age 48. No other cancers were reported in his second degree relatives. A HNPCC cancer, stomach cancer was reported in a paternal cousin at age 60. Three breast cancers and a lung cancer were reported in paternal cousins. A breast cancer was reported in a maternal cousin. No other cancers were reported in this family.

MSS and hMSH6- (n= 1)

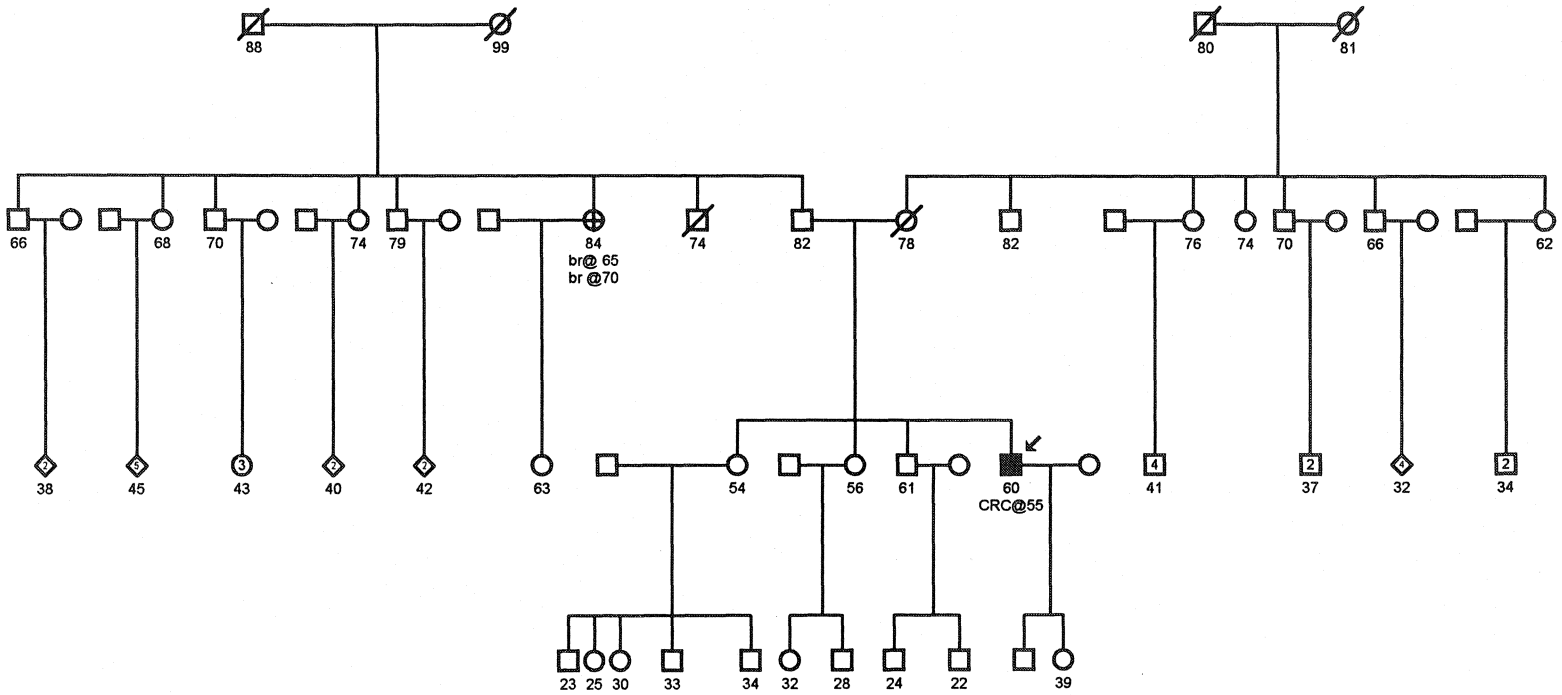
Proband number 119 (pedigree 32) is a 69 year old male who was diagnosed, in 1997, with a well differentiated cancer of the sigmoid colon at age 64. He was born in Arnolds Cove and he now resides in Green Harbour. His mother and his maternal grandparents originated from Woody Island, Placentia Bay. His father and his paternal grandparents originated from Arnolds Cove. The pedigree constructed consists of 39 relatives. He has 5 first degree relatives: 2 children, 1 sibling and parents. His mother died from arthritis complications at age 82, and his father died from heart complications at age 61. His sibling died of heart complications at age 67. No other cancers were reported in his first degree relatives. He has 15 second degree relatives. A skin cancer was reported in his maternal grandfather who died at age 92 and a prostate cancer was reported in a maternal uncle who died at age 92. No other cancers were reported in this family.

MSS and no loss (n = 15 families)

See appendix A, pedigrees 51 through 65

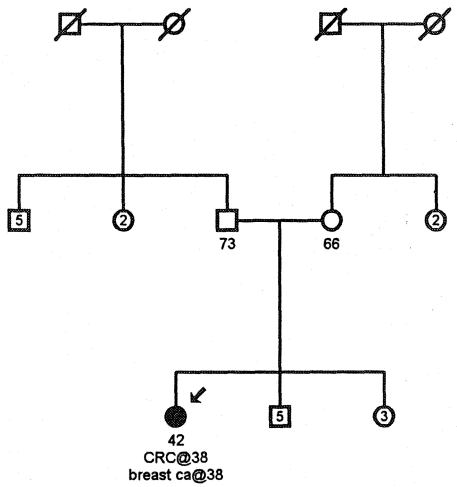
Pending laboratory results (n=2)

See appendix A, pedigrees 66 through 67



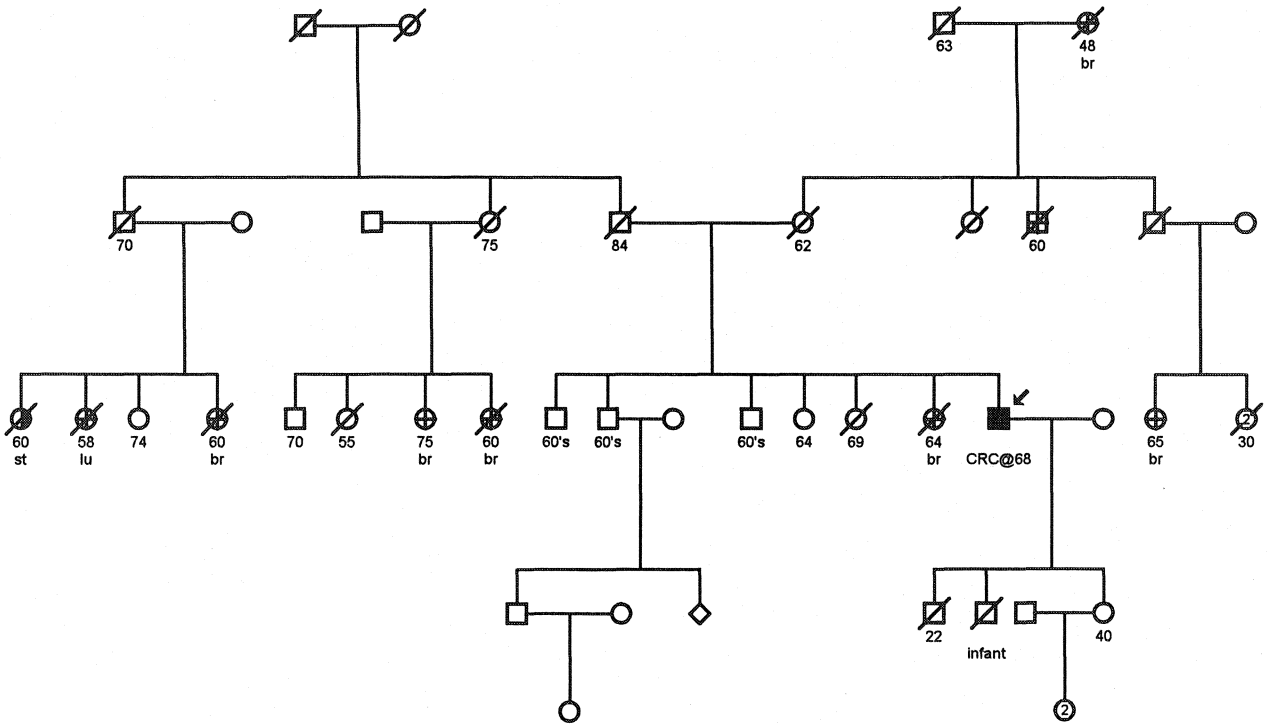
Pedigree 29 Low-Family 108

MSI
MLH1-



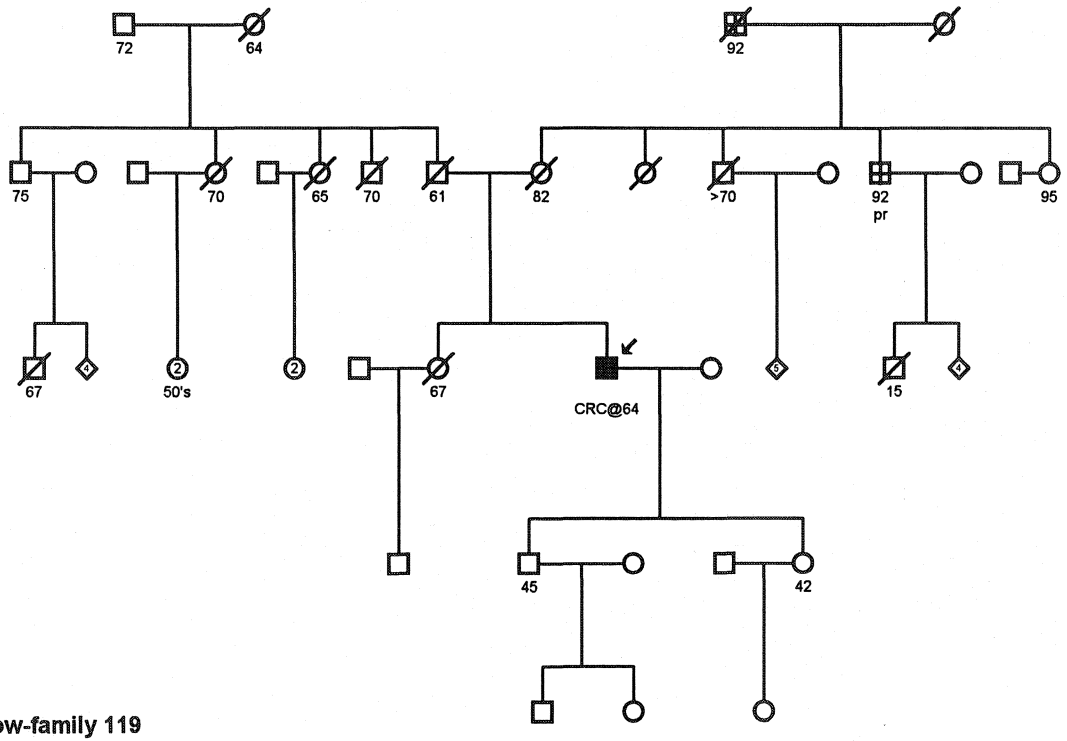
Pedigree 30 Low-Family 189

MSI
MSH6-



Pedigree 31 Low-Family 63

MSI
no loss



Pedigree 32 **Low-family 119**

MSS
MSH6-

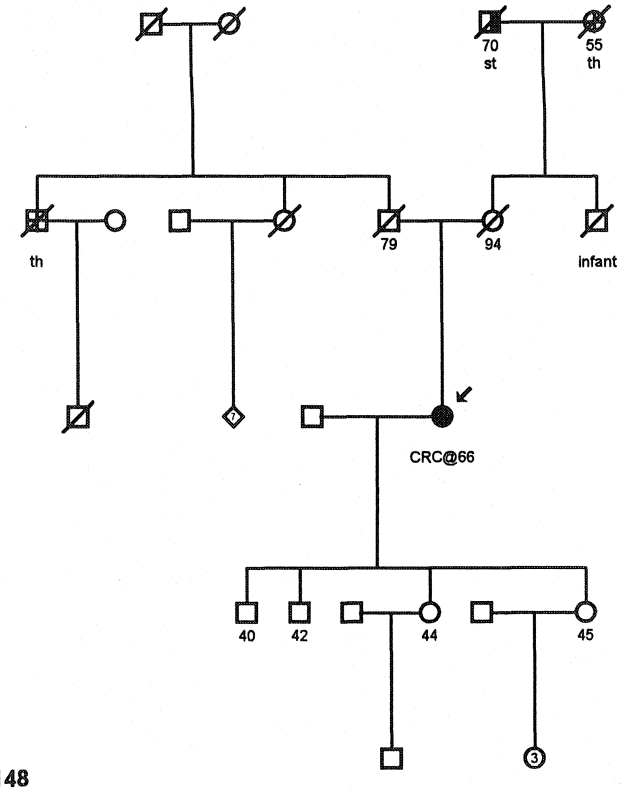
LOW RISK – Uninformative (n= 13 families)

MSI-H and hMLH1- (n=1 family)

Proband number 148 (pedigree 33) is a 71 year old female who was diagnosed, in 1997, with a moderately differentiated cancer of the hepatic flexure at age 66. Chart review indicated the presence of 2 tubular adenomas in 1998. She was born in Cupids and now resides in St. John's. Her mother and her maternal grandparents originated from Cupids. Her father and her paternal grandmother originated from Cupids. She is unaware about the origins of his paternal grandfather. The pedigree constructed consists of 25 relatives. She has 6 first degree relatives: 4 children, and parents. Her mother died an accidental death at age 84. Her father also died an accidental death at age 79. There were no reported cancers in her first degree relatives. She has 11 second degree relatives. A HNPCC cancer, stomach cancer, was reported in her maternal grandfather who died at age 70, and a throat cancer was reported in her maternal grandmother who died at age 55. A throat cancer was reported in a paternal uncle (age unknown). No other cancers were reported in this family. The family is uninformative because there are less than six siblings at risk.

MSS and no loss (n = 12 families)

See appendix A, pedigrees 68 through 79.



Pedigree 33 Low uninform-Family 148

MSI
MLH1-

CHAPTER 4: DISCUSSION

This study has provided information about hereditary CRC on the Avalon Peninsula of Newfoundland and Labrador. Since there was no single defining feature, family history was the most important tool in making a HNPCC diagnosis. However, the heterogeneous nature of HNPCC hinders the diagnosis. Diagnosis is further complicated because CRC and other HNPCC cancers most frequently occur as sporadic tumours, because of the small size of most of today's families, and because individuals may die young of other causes before they develop the cancer. Individuals may also have limited knowledge about their families, so extensive pedigrees and family histories are not usually available in clinical practice (Vasen et al, 1999). Furthermore, when the criteria are used strictly, non-penetrance of cancer in a mutation carrier may prevent a HNPCC diagnosis.

Advances in the understanding of molecular genetics of CRC have led to another method of diagnosis. We now know that individuals who harbour mutations in mismatch repair genes are at increased risk for a HNPCC cancer (Peltomaki and de la Chapelle, 1997). Therefore this condition can now be identified by 2 different methods: clinical or molecular. Some individuals have both clinical and molecular evidence of the condition and such an overlap is a positive factor towards increasing the confidence in the diagnosis.

The prevalence of HNPCC in the Newfoundland population was evident with potentially 41 families, previously identified through the Medical Genetics program, fulfilling Amsterdam Criteria I (AC I) or Amsterdam Criteria II (AC II). The occurrence

was further confirmed when a point mutation, A→T at nucleotide 943 + 3 in the 3' splice site of exon 5 was identified in affected members of one family. This mutation ("family C") results in the deletion of exon 5 and a truncated protein. So far this mutation was found to segregate among 13 families with at least 176 individuals testing mutation positive.

Early studies (elsewhere) concentrated on high risk individuals to estimate the prevalence of HNPCC. These studies reported that 3.8-5.5% of CRC patients had HNPCC. Subsequent studies suggested that this was probably much higher than the overall population risk because of the biased sample used. However using a retrospective population-based approach we identified 22 of 78 families (28.2%) at high risk (HR) and 22 families (28.2%) at intermediate risk (IR) for HNPCC in the Eastern region of Newfoundland. The criteria used to categorise these families was assembled from the Amsterdam Criteria I (AC I), the Amsterdam Criteria II (AC II), and the Ontario Familial Colorectal Cancer Registry (OFCCR).

Comparison With Other Studies

Different study designs prevent comparison of several major population-based studies. Several groups have reported the frequency of HNPCC by using the AC I and other less strict HNPCC criteria. By the AC I, Mecklin et al (1995) reported 0.7% frequency of HNPCC in the Finnish population, Evans et al (1997) reported 0.3% frequency of HNPCC in a British population, Katballe et al (2002) reported a 0.9% frequency of HNPCC in the Danish population. By comparing our study to these studies we found that a total of 7.7% of our families (6 families) met the AC I. Using less strict

criteria these same authors reported the frequency of HNPCC to be 1.7% (Mecklin et al, 1995), 1.4% (Evans et al, 1997), and 2.3% (Katballe et al, 2002). Using less strict criteria we once again report a higher frequency of HNPCC, i.e. 56% of our families (44 families). Differences in each study's less strict criteria may account for different results, though the criteria used by Evans et al (1997) are similar to ours. Population differences may also account for the different results. For example, the founder effect in the Finnish population may increase the HNPCC incidence in Finland. This could also be the case in our study due to our founder mutation ("family C") in one of our probands (# 122, pedigree 7).

Today, most population-based studies attempt to determine the frequency of HNPCC by molecular detection. This molecular detection begins with Microsatellite instability (MSI) analysis of consecutive CRC cases. Those cases that are MSI-H are screened for germline mutations usually of the hMSH2 and hMLH1 genes (Aaltonen et al, 1998; Salovaara et al, 2000; Katballe et al, 2002). More recently immunohistochemistry (IHC) analysis to test for the protein expression of the MMR genes (particularly hMLH1, hMSH2, and hMSH6) has been added (Percesepe et al, 2001). A combination of these molecular methods and family history criteria are used to estimate HNPCC frequencies in various studies. We chose to study each case with MSI analysis and IHC analysis, the results of which will be discussed later.

The importance of utilising the AC II in our study criteria is demonstrated by the participation of a mutation positive proband. Proband 122 (pedigree 7) is a carrier of the "Family C" mutation. However, the proband's family does not meet the AC I because of infrequent CRC and frequent extracolonic cancers. There are only 2 individuals with

CRC and these are not first degree relatives. However the proband's mother is mutation positive and is affected with a double primary of endometrial and ovarian cancer. Many individuals in this family have not agreed to be investigated with clinical screening therefore it is possible that other individuals are affected.

We further modified the AC by extending the threshold for youngest age of cancer to age 60, and also to include several other HNPCC cancers to make our 3rd HR group. This was called the "Age and Cancer Modified Amsterdam Criteria". The decision to increase the age range was based on a recent study on hMSH2 mutation carriers in the Newfoundland population (Green et al, 2002). Results of this study showed that a significant number of first cancers occurred after age 50. The cumulative risk of first cancer went from 72% at age 50 to 86% at age 60. Family 50 (pedigree 13) is an example of a family meeting our Age and Cancer Modified Amsterdam Criteria. CRC is observed in five individuals in two consecutive generations all over the age of 50, however one individual is diagnosed at 51, and another is diagnosed at age 53.

The decision to add more cancers to the diagnostic criteria for the 3rd HR group was based on the high relative risk of these cancers in HNPCC as reported in the literature (Mecklin et al, 1991; Watson et al, 1993; Aarnio et al 1995; Vasen et al, 1996a; Dunlop et al, 1997). The cancers selected, for the 3rd HR group, are those proposed by the International Collaborative Group on HNPCC and those cancers chosen by the OFCCR group. Family 183 (pedigree 12) is an example of a family we considered HR by the Age and Cancer Modified Amsterdam Criteria. There were 14 HNPCC cancers (including endometrial, pancreas, gall bladder, stomach, ovarian) reported in this family.

The OFCCR group included lymphomas in their intermediate criteria. The rational for including lymphomas comes from the development of lymphomas in HNPCC Knock-out mice. These mice form skin neoplasms (analogous to Muir-Torre syndrome) and also develop lymphomas (de Wind et al, 1999). The lymphoma was included as a HNPCC cancer in 2 families in this study. However it will not be included as a HNPCC cancer for the development of the NFCCR database as its significance in humans has not been clarified (Teruya-Feldstein et al, 2002). Other cancers are reported to occur in HNPCC. For instance we did not include bladder cancer as a HNPCC cancer. However, recently a new mutation (hMSH2 deletion exon 8) was identified in Newfoundland families (Jane Green, personal communication). A defining clinical feature in these families was transitional cell cancers of the renal pelvis, ureter and bladder.

MSI and IHC analysis correlated extremely well. Fifteen of sixteen MSI-H cases (93.8%) had loss of expression of one or more proteins. One MSI-H case (pedigree # 31), which was classified as LR, did not show loss of protein expression. Several explanations may explain this finding. An area of uncertainty exists when interpreting IHC results. It is possible that the interpretation of the IHC analysis was incorrect and there actually was loss of protein expression. Even though the family history does not suggest inherited disease, it may be feasible that a missense mutation, occurring at the COOH terminus of the protein, results in expression of a non-functioning protein (R. Kolodner, personal communication in de la Chapelle, 2002). Or perhaps a new mutation exists in this individual and the defect lies in one of the MMR genes not investigated in this study.

MSI and IHC analysis also did not correlate when two tumours (family 7, pedigree 25 and in family 119, pedigree 32) had MSS by microsatellite instability

analysis and did not express the hMSH6 protein. Mutations of hMSH6 with MSS by microsatellite instability analysis are previously reported in the literature (Wu et al, 1999). Family 7 is HR for HNPCC by the AC 1. The proband's family meets the criteria because several of his 2nd degree relatives have CRC and other HNPCC cancers (e.g. kidney, pancreas). The proband's mother (diagnosed with breast cancer at age 67 and died at age 75) was probably an obligate carrier as HNPCC is known to have a reduced penetrance (Lynch et al, 1996a). Other variables might affect the phenotypic expression of a certain mutation such as gene polymorphisms, modifier genes, and other environmental factors. For instance, patients who have HNPCC with a common polymorphism in the cyclin D1 gene developed CRC eleven years earlier than patients who did not carry the polymorphism (Kong et al, 2000). Unlike family 7, there are no other HNPCC cancers reported in family 119. Furthermore, endometrial cancer, which is reported to be common in hMSH6 families, was not reported in these families. Further study of the probands and their family members is warranted.

Another proband (# 189, pedigree 30) where the tumour did not express the hMSH6 protein was MSI-H. This individual (included in the LR group) was previously referred to the Medical Genetics program because she had a double primary of CRC and breast cancer at age 38. The significance of the breast cancer in this individual is unknown. It may be important to explore the stability and protein expression in the breast cancer tumour tissue. The proband reported no other cancers in the family. As she originates from another country it is possible that the reported information is incorrect as she may not be aware of her family history.

In this study 54 of 71 CRC cases (76.1%) had MSS tumours and expressed all three proteins (hMLH1, hMSH2, and hMSH6). An MSS result by MSI analysis suggests non-HNPCC. Our proportion of MSS tumours is lower than reported by other population based studies. Salovaara et al (2000) reported MSS in 88% and Percesepe et al (2001) reported MSS in 91.7% of their populations. Twenty-seven of our 54 MSS cases (50%) were classified as LR on review of their family history. These cases are almost certainly sporadic. However 24.1% of MSS cases (all 13 cases expressed all proteins) were HR by family history and in fact three of these cases met the AC 1. Peel et al (2000) reported MSS and no mutations (hMLH1 and hMSH2 genes) in 5 of 9 HNPCC families. In a Danish population-based study, mutations were found in 2 of 6 AC I families therefore it was suggested that mutation negative individuals from HR families have a 25% chance of meeting the AC I (Katballe et al, 2001a). The possibility exists that there are yet other undiscovered genes responsible for hereditary CRC and HNPCC.

Germline mutations in other genes, besides the MMR genes, may be causing the cancers or a non-genetic clustering of CRC may be represented by various HNPCC criteria. Non-genetic clustering of cancers cannot be ruled out for both the HR and IR groups showing MSS on MSI analysis. However, this is unlikely in some families, for example the AC 1 family 35 (pedigree 4). Extension of this family reveals cancers in multiple generations and individuals and therefore it is very unlikely that non-genetic clustering occurs in this family. It is important to screen other tumours in this family by MSI analysis to determine if other clinically affected family members have the same molecular findings as the proband. If MSI-H tumours are found it is probable that our

proband is a phenocopy. If MSS tumours are found then another gene is undoubtedly responsible for the cancers in this family.

In our population MSI-H tumours were found in 16 (21.6%) of 74 cases. This figure is higher than reported in other population based studies. Salovaara et al (2000) reported 12% and Percesepe et al (2001) reported 8.3%. One of our families, that is HR by the AC I, contains two probands, 11 (pedigree 1) and 12 (pedigree 2), that are first cousins. MSI analysis revealed that both probands were MSI-H, and IHC analysis demonstrated that neither tumour expressed the hMSH2 protein. The cousins are members of a high risk family already known to the Newfoundland Medical Genetics program.

Recently, as part of an ongoing project, Dr. Michael Woods (personal communication) studied a number of the previously identified families in this cohort. His work includes the search for mutations. Several interesting findings, pertaining to this study, were made while using a fairly new method, multiplex ligation-dependent probe amplification (MLPA), to search for genomic deletions. First, the mutation segregating in family 11 and 12 was identified. This is a deletion of exon 8 of the hMSH2 gene. This mutation has now been determined to segregate in 6 families. Second, MSI analysis and IHC analysis results between this study and Dr. Wood's study are consistent. Dr. Woods also reports an alteration in the hMSH6 gene (G1139S) in proband 45. This alteration has not been reported previously and its pathological significance is unknown at this time. It is interesting to note that IHC analysis showed loss of expression of both the hMSH6 and hMSH2 proteins. It has been postulated that the loss of expression of hMSH6 may be secondary to that of mutations in hMSH2 (Cunningham et al, 2001; Planck et al, 2000;

Wu et al, 1999) because of the physical proximity of hMSH2 and hMSH6. However the alternative, loss of expression of hMSH2 due to a mutation in hMSH6 has not been reported. Further studies may lead to a better understanding of this factor. We chose to alter the criteria when considering family 45. This individual was given an IR classification (IR #2). However, there are not 3 cancers in 3 individuals in the family as required for inclusion in the IR group. Instead, for this case the criteria are interpreted to include 3 cancers in 2 individuals. It is possible that a new mutation is responsible for the cancers in this family, that the history given is incomplete, or that the small family size resulted in fewer affected, even in a true HNPCC family. It is also possible that a maternal grandparent is an obligate carrier. Non-penetrance has been demonstrated to play a role in HNPCC and it has been estimated that approximately 10% of carriers will never develop a cancer (Dunlop et al, 1997).

Another family (#135, pedigree 27) where the hMSH2 and hMSH6 proteins were not expressed was MSI-H and is IR. This proband was classified as IR for HNPCC because a maternal cousin had CRC at age 35. The proband also reported that his mother possibly had a cancer. Efforts to review the mother's records are on-going. If she did have a HNPCC cancer, then this family will be reclassified as HR. The "family C" proband (# 122, pedigree 7) also had a loss of expression of the hMSH2 and hMSH6 proteins. At the time of the discovery of the "family C" mutation the significance of the hMSH6 gene was not known and IHC analysis was not used for the detection of MMR proteins. It would be interesting to investigate whether all mutation positive "family C" individuals fail to express the hMSH6 protein.

Seven (43.8%) of the 16 MSI-H cases were deficient for the hMLH1 protein. Two of these were from the IR group and two from the LR group. It is probable that a number of these cases will be explained by the epigenetic mechanism of hypermethylation. In a study by Percesepe et al (2001) 14 of 20 cases (70%) that were MSI-H and did not express the hMLH1 protein were hypermethylated. Hypermethylation analysis will be performed on the promoter region of hMLH1 for cases which showed MSI-H, with loss of hMLH1 expression.

Study Difficulties

Several studies (Aitken et al, 1985; Love et al, 1985; Sijmons et al, 2000) concluded that the diagnosis of cancer reported by first degree relatives is very likely to be accurate (77-93%). The use of population-based registries, HNPCC registries, and cancer registries enhance verification and ensure a more accurate estimate of those at risk. A recent Danish population based study (Katballe et al, 2001b) using their specialised registries indicated that CRC was reported correctly 68.4% of the time in first degree relatives. Newfoundland does not have the same population registry or existing specialised registry. Furthermore access to records for research purposes is restricted by privacy laws that are enforced by ethics boards and consequently our ability to confirm information was limited. Permission to confirm reported cancers through the Newfoundland Cancer Treatment Research Foundation (NCTRF) registry was granted to ease the verification process. By using names, birth dates, and place of residence, we identified 207 reported cases. Of these only 21 discrepancies were identified giving us a false positive rate of 10.1%. However no information is available about the other

reported cancers (n = 229). The MCP (provincial medical ID) identity number is the only way to confidentially ensure that the correct identity was used. This number was not available to us without consent from the affected person. For the current NFCCR study, the NCTRF registry will be used only after consent has been obtained from the affected family member or from a next of kin if the affected is deceased. Access to the registry will ease the verification process, as the ability to use such a resource has proved invaluable in other population studies.

It is important to verify the reported cancers histologically as this confirms the diagnosis which is a requirement of the AC. In this study all CRC, except one, in relatives in the AC I and AC II families were confirmed. The cancer not confirmed is in a sibling of the proband who lives in a different province. Retrieval of the records and a tumour specimen is in process. All of our AC HR group were previously referred to the medical genetics program because of suspected hereditary cancer. This might overestimate the frequency of HNPCC in our population because it is probable that those associated with a genetic clinic are more likely to participate in a research project as personal benefit is possible. However, this is not always the case as some individuals do not want to go through the process again so this could balance out those who were more likely to participate. For example we are aware of several individuals, including a mutation positive ("family C") individual, previously seen by the genetics program who opted not to participate. We also avoided an ascertainment bias by recruiting all participants in the same manner.

The study by Katballe et al (2001) reported a false negative family history reporting rate of 39%. In their study, 7 of 18 AC I and AC II families were initially

classified incorrectly and it was only after further investigation that the classification of AC I and AC II were given. False negative reporting of family history was also observed in our study. Two HR probands, family 7 (pedigree 3) and family 35 (pedigree 5) did not report several CRC in their families which initially resulted in a LR classification. However their families were previously referred to the medical genetics program and the CRC in question were documented. It is probable that more AC families may be identified from further follow-up of the other LR and IR families. This follow-up will happen when these probands attend genetic counselling. It is at this step that records will be collected for cancer verification which is necessary to recommend appropriate screening in those with known and unknown mutation status. It will only be then that current risk classification will be verified.

Germline mutations have been found in families not fulfilling the AC (Nystrom-Lahti et al, 1996; Moslein et al, 1996; Wijnen et al, 1997; Genuardi et al, 1998). An inherent misclassification bias may exist when diagnosing a condition on the basis of family history alone, particularly in small families. The issue of family structure and family size has been addressed for assessing breast cancer risk. BRCAPRO is a statistical computer program that calculates probabilities of mutations in the known breast cancer susceptibility genes, BRCA1 and BRCA2, based on family history alone (Parmigiani et al, 1998; Berry et al, 2002). A program of this nature has not been developed for assessing HNPCC risk. Therefore, we chose to determine the informativeness of the low risk families. Our definition of informativeness was our preliminary effort to acknowledge the restrictive nature of the clinical diagnosing criteria. The definition was based on how many potential first and second degree relatives, in 2 generations, are at

risk for developing HNPCC. Since we proposed that there need be at least six at risk relatives (of the proband and/either parent) and since age 60 was our HR age threshold we used this as our cut off age for informativeness. Thirteen families (38.2%) were classified as uninformative, because there were too few and/or too young family members or insufficient family details. A number of families were suggestive of HNPCC but a lack of family details made it impossible to classify them as IR or HR. For example the information reported for family 164 (pedigree 77) was given by the proband's wife, and she was unaware of the proband's second degree relatives. Furthermore the available family history for family 65 (pedigree 68) is not sufficient to state that the family is not at risk for HNPCC. For this case the participant agreed to participate in the study but did not disclose any further information such as dates of birth and ages of death for his relatives.

The utility of testing for MSI and MMR protein expression for the diagnosis of HNPCC in this population was poor. Only 30.0% of our HR families were picked up by MSI analysis. If we calculate the sensitivity of MSI analysis to detect AC and AC II families it increases to 33% in our population. This sensitivity is much lower than reported by Katballe et al (2002). They report a 61% sensitivity of MSI analysis to detect AC I and AC II families. However they report a 100% sensitivity of MSI to detect genes carriers. Therefore MSI is a sensitive tool for the identification of mutations in the MMR genes. This suggests that MSI analysis is a useful tool for detection of mutations in known genes but unreliable for the detection of AC families. However it may be a useful tool to rule out LR HNPCC families. In this study 84% of our LR families were MSS by

MSI analysis. It is important to remember that the original purpose of MSI analysis was to identify cases in which to find mutations in the MMR genes.

Lack of a uniform disease makes it difficult to assess the sensitivity, specificity, and predictive powers of the laboratory methods. Therefore, the question remains, will there be a need for both MSI and IHC analysis? Combining the results of both techniques is sometimes difficult as the use of both a histology lab and molecular lab are needed. MSI analysis is more costly, time consuming and more tissue is needed for a result, while IHC analysis directs the search for specific germline mutations, costs less, and is less time consuming (de la Chapelle, 2002). A criticism of the use of IHC analysis alone is the possibility that some mutations may not be detected because they may result in expression of a protein even though it is not functioning. As previously mentioned, missense mutations may inhibit a protein's function but still allow IHC expression (de la Chapelle, 2002). As IHC analysis is a new technique its value is yet to be determined. No general consensus exists on whether MSI analysis, or IHC analysis, or both will be recommended in future.

All of these efforts may answer many important questions such as how this information will be used, who will benefit, and who in the general population should be screened. Predictive testing for adult onset disorders could lead to preventing conditions. In some cases this testing may relieve unnecessary worry while in others it may increase anxiety and feelings of guilt (Huggins et al, 1992; Tibben et al, 1993), and for others may introduce potential for discrimination and stigmatization (Aktan-Collan et al, 2000). The safety, effectiveness, and quality of these tests must be evaluated. Screening should be tailored to the presentation of cancers occurring in the family. In the future it may be

possible to determine through genetic testing what the presentation will be like. One on one counselling may increase acceptance of predictive testing, and the use of prophylactic surgeries to keep a person cancer free at target organs, may be warranted in mutation positive individuals.

Limitations

A low number of subjects (50% of those eligible) enrolled in this study. As this was a pilot study, limited resources may have contributed to factors influencing enrolment such as a delay before follow-up after the letters were sent out and delays of ethics approval for amendments (Human Investigation Committee). It was not possible to collect reasons for non-participation because of the opt-out option of our study design. The retrospective study design probably contributed to the low uptake as three to four years had passed since the subjects were diagnosed with their CRC. It was possible that some individuals were ill, deceased, or did not want to revisit a painful time in their lives. Several changes to the design of our study and several amendments to ethics approval contributed to an easier transition for development of the NFCCR. Also the hiring of a professional staff will lead to a more accurate assessment of hereditary HNPCC in Newfoundland possibly by improved methods of ascertainment under strict timelines. The latest statistics released from this group indicated a 52% (of those eligible) enrolment of CRC probands. However there are many cases in process.

Due to the heterogeneous nature of HNPCC the ascertainment of only CRC incident cases is limiting for its identification. This may underestimate the total prevalence of HNPCC in our population. Several studies have shown that the risk of

endometrial cancer is higher than the risk of CRC in female mutation carriers (Aarnio et al, 1995; Dunlop et al, 1997; Aarnio et al, 1999; Green et al, 2002). Even though only CRC was used as index cases it is necessary to establish the resources before registration begins. This can then be expanded to include other tumours, such as endometrial tumours in the future.

For this study records of screening and interventions were not obtained. It is possible that individuals within each family had precancerous lesions (adenomas) removed, thus altering the natural history of HNPCC or CRC in these families. Also preventative surgeries such as hysterectomies could prevent the expression of other cancers. New criteria need to be developed and implemented to capture these families as they could easily be missed when identifying HNPCC families. The Bethesda Criteria does recommend MSI analysis in CRC probands who have first degree relatives with adenomas at less than 40 years and also in individuals who have adenomas at less than 40 (Rodriguez-Bigas et al, 1997). However these criteria are currently not used in clinical practice in Newfoundland.

We do not know the proportion of mutations in our group. This study did not complete mutation analysis on any individual. However, because numerous subjects were under the care of the medical genetics program the mutation status of some individuals was known. We did not stain for proteins hPMS1, hPMS2, and hMSH3, because the antibodies for these agents were not commercially available at the beginning of our study. Moreover our resources were limited, and because mutations in these genes appear less common they were excluded.

Future Research

Hypermethylation analysis will be carried out on the promoter region of hMLH1 for cases which showed MSI-H, with absence of expression of hMLH1. We did not report MSI-L tumours. Instead we reported MSI-L tumours as MSS. There were 4 MSI-L cases in this series (families 68, 133, 137, 166). It is possible that MSI-L tumours have a more distinct phenotype than MSS tumours. For example tumours in MSH6 knockout mice may display an MSI-low phenotype (Edelmann et al, 1997). Several tissue samples were not available to us. Efforts to obtain them are underway. If it appears impossible to obtain them, then other tumours within the family will be obtained for molecular investigation as is currently the case with an AC family 157 (pedigree 7).

In suspicious families attempts will be made to determine if tumours are not just phenocopies, and further contact will be made with family members in order to obtain medical records and extend pedigree information. The determination of consistent molecular characteristics in all affected family members could lead to an accurate diagnosis of HNPCC. We did collect blood samples (DNA), where possible, at the time of interview. A search for known mutations will be carried out on cases showing absence of protein expression. Linkage analysis will be performed on all HR and IR families where MSS was found. However this is a difficult task because of lack of DNA from deceased affected family members, and difficulties arising classifying family members because of variable expression and reduced penetrance of HNPCC genes. Estimates of cancer risk in these families will be calculated and will be the subject of further research.

Hormonal influences may influence the phenotypic explanation of gender differences (Froggatt et al, 1999; Dunlop et al, 1997). These differences may account for

the different phenotypes in males than females. This area of interest is being studied by a PhD student involved with the CRC-IHRT project. In addition, the NFCCR will evaluate psychosocial aspects of CRC, lifestyle and dietary habits of CRC cases.

It may be possible to test for MSI in the general population; however this pilot study could not effectively determine its value as a screening test for the detection of HNPCC. Further population based studies with larger numbers are needed to interpret MSI analysis in order to improve its potential use as screening test for HNPCC.
In future, the significance of such findings may lead to improved stratification of patient treatments, such as chemotherapy, and potentially gene therapy. It seems likely that improvement in the molecular genetics in CRC will also be applied to other cancers as well.

Summary

The present study concluded that the risk for HNPCC is high in the Eastern Newfoundland population. Nine percent of families (n=7) are at high risk for HNPCC, by the Amsterdam I and II criteria. However, 3 of these families had MSS tumours and the tumours expressed all proteins. It is likely that there are unknown MMR repair genes or other genes responsible for HNPCC in this population. Another 37 families (47%) are also at higher risk for HNPCC by less strict criteria. Furthermore 5 LR families warrant further investigation because of molecular findings. To prevent overestimating HNPCC in this population a worse case scenario analysis was completed. If we assume that all non-responders (n=100 or 56%) belong to non-Amsterdam Criteria families we could say that the potential frequency of Amsterdam Criteria I and II families is 7/178 or 4%, and

the potential frequency of families at higher risk for HNPCC is 37/178 or 21%. Therefore the potential range in frequency of Amsterdam Criteria families is between 4 and 9%, and the potential range in frequency of families at higher risk for HNPCC is between 21 and 47%.

A combination of family history, microsatellite instability analysis, and immunohistochemistry analysis appear to be a valuable diagnostic tool in the search for mutations and also valuable for the identification of families that may warrant further exploration. In the near future, there will likely be strict clinical and pathological guidelines for testing specimens. A study of this nature demonstrates the need for a multidisciplinary approach to the identification and care of HNPCC families, as it involves the collection of a vast amount of information and would be difficult for any one discipline. It is difficult to draw any major conclusions because of the low number of families in our study. Therefore, further studies, such as the CIHR-IHRT study, should be performed to improve evaluation of the significance of our findings. The development of a specialised Newfoundland familial CRC registry is currently on going. Its development will take 5 or more years and will identify the true incidence of HNPCC within the Newfoundland population so that appropriate resources will be developed for its future identification and management.

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APPENDIX A: Family descriptions and pedigrees (#'s 34-79)

INTERMEDIATE CRITERIA 1 (n= 11)

MSS and no loss (n = 8 families)

Proband number 2 (pedigree 34) is a 67 year old male who was diagnosed, in 1997, with a moderately differentiated cancer of the transverse colon with liver metastasis at age 62. Pathology at the time of the surgery revealed the presence of 10 villo-nodular and tubular polyps. He also had a prostate cancer at age 63 and a melanoma at age 54. The proband was born in Kingwell, Placentia Bay and he now resides in Little Harbour. His mother and his maternal grandfather originated from Kingwell, Placentia Bay. His maternal grandmother originated from Hopeall. His father and paternal grandparents originated from Kingwell, Placentia Bay. The pedigree constructed consists of 46 relatives. He has 9 first degree relatives: 1 child, 6 siblings and parents. His mother died of a HNPCC cancer, stomach cancer, at age 71. His father was diagnosed with a throat cancer at age 69 and he died at age 81. No other cancers were reported in his first degree relatives. He has 24 second degree relatives. Three HNPCC cancers were reported in second degree relatives. A liver cancer was reported in the maternal grandmother. A stomach cancer was reported in a paternal aunt, who died in her 70's, and a liver cancer in his paternal grandfather who died in his 60's. A bladder cancer was reported in a maternal aunt who died at age 78, a cancer (type unknown) was reported in his paternal grandmother, who died in her 60's and another cancer (type unknown) in a paternal uncle. No other cancers were reported in the family.

Proband number 16(pedigree 35) is a 63 year old male who was diagnosed, in 1997, with a moderately differentiated cancer of the sigmoid colon at age 57. He was diagnosed with a second primary of the colon in 1999. A chart review revealed 4 benign

polyps and a hyperplastic polyp on follow-up colonoscopy in 2001. He was born in South River and now resides in Manuels. His mother and maternal grandfather originated from Clarke's Beach. His maternal grandmother originated from Brigus. His father and paternal grandfather originated from South River. His paternal grandmother originated from Cupids. The pedigree constructed consists of 43 relatives. He has 10 first degree relatives: 5 children, 3 siblings, and parents. A brother was diagnosed with a HNPCC cancer, CRC, at age 63. His mother died at age 84 from natural causes. His father died at age 71 from complications of atherosclerosis. A brother died from prostate cancer metastasis at age 64. No other cancers were reported in his first degree relatives. He has 25 second degree relatives. Three HNPCC cancers were reported in second degree relatives. Endometrial cancer was reported in two maternal aunts at ages 78 and 83, and stomach cancer was reported in a maternal uncle at age 72. A paternal aunt was diagnosed with cervical cancer and she died at age 61. No other cancers were reported in the family.

Proband number 21(pedigree 36) is a 67 year old male who was diagnosed, in 1997, with a moderately differentiated cancer of the splenic flexure at age 62. This was his second primary. His first primary was a cancer of the rectum in 1995. Chart review revealed a history of 6 polyps showing a tubular adenoma configuration. He was born in New Melbourne, Trinity Bay and now resides in Brownsdale. His mother and paternal grandmother originated from New Melbourne. His maternal grandmother originated from New Chelsea. His father and paternal grandparents originated form New Melbourne. The pedigree constructed consists of 35 relatives. He has 5 first degree relatives: 1 child, 2 siblings, and parents. His mother died from childbirth at age 18. His

father died at age 65 accidentally. No cancers were reported in his first degree relatives. He has 22 second degree relatives. There were four HNPCC cancers reported in second degree relatives. A HNPCC cancer, CRC, was reported in his maternal grandmother who died at age 88 and a cancer (type unknown) was reported in maternal grandfather who died at age 69. HNPCC cancers, stomach cancers were reported in both paternal grandparents and a paternal uncle. Another paternal uncle was diagnosed with a prostate cancer and died at age 50. No other cancers were reported in the family.

Proband number 44 (pedigree 37) is a 70 year old female who was diagnosed, in 1998, with a moderately differentiated cancer of the hepatic flexure at age 66. Chemotherapy was required. She was born in Brigus South. Her parents and grandparents originated from Brigus South. The pedigree constructed consists of 76 relatives. She has 15 first degree relatives: 4 children, 9 siblings and parents. Her daughter died at age 36 from cervical cancer. Her sister was diagnosed with a breast cancer at age 59. Her mother died at age 85 from Alzheimer's and her father died at age 91 from a stroke. There are no reported cancers in her first degree relatives. She has 35 second degree relatives. There were three HNPCC cancers reported on the paternal side of her family. They included a CRC in a paternal uncle at age 68, a stomach cancer in a paternal aunt at age 49, and a stomach cancer in a paternal cousin (age unknown). Other cancers reported include a lip cancer and a breast cancer in 2 paternal cousins and a cancer (type unknown) in a paternal uncle. No other cancers were reported in the family.

Proband number 72 (pedigree 38) is a 62 year old female who was diagnosed, in 1997, with a moderately differentiated cancer of the descending colon at age 57. She was diagnosed with liver metastasis in 1998, again in 1999, and then finally

metastasis to the lung in 2001. She was born in Dildo, Trinity Bay and now resides in Mt. Pearl. Her mother originated from Thornlea. Her maternal grandparents originated from Trinity Bay although the exact location was not given. Her father and paternal grandfather originated from Dildo, Trinity Bay. Her paternal grandmother originated from Norman Cove. The pedigree constructed consisted of 80 relatives. She has 14 first degree relatives: 4 children, 8 siblings, and parents. Her mother died at age 53 from metastasis to her spine form a HNPCC cancer, kidney cancer. A sister was diagnosed with breast cancer at age 55. No other cancers were reported in first degree relatives. Polyps were reported in three siblings. Crohn's was reported in two siblings. One child was scoped. Her father died at age 52 from complications of asthma. She has 33 second degree relatives. Three HNPCC cancers were reported in second degree relatives. A paternal aunt was diagnosed with CRC at age 63, her paternal grandfather was diagnosed with CRC before age 68, and a paternal uncle was diagnosed with a stomach cancer before age 62. Another paternal uncle was diagnosed with a lung cancer, age unknown. A paternal aunt was diagnosed with a skin cancer, age unknown. A cancer, unknown type, was reported in a maternal aunt in her 80's. No other cancers were reported in second degree relatives. A maternal cousin was diagnosed with prostate cancer at age 62, and another maternal cousin was diagnosed with lung cancer. Two maternal cousins are diagnosed with breast cancers, age unknown. No other cancers were reported in the family.

Proband number 126 (pedigree 39) is a 58 year old male who was diagnosed, in 1998, with a moderately differentiated cancer of the rectum at age 54. Chemotherapy and radiation were required. Past history reveals the presence of polyps (no details given).

He was born in Colliers where he now resides. His mother originated from Colliers. His paternal grandfather originated from Colliers and his paternal grandmother originated from Conception Harbour. He has 17 first degree relatives: 15 siblings, and parents. His mother died at age 74 from pneumonia and his dad died at age 57 from heart complications. A brother has crohn's. He has 31 second degree relatives. Two HNPCC cancers are reported in his maternal family. A maternal uncle was diagnosed with CRC at age 73, and his maternal grandmother was diagnosed with stomach cancer at age 82. No other cancers were reported in the family.

Proband number 112 (pedigree 40) is a 64 year old female who was diagnosed, in 1997, with a moderately differentiated cancer of the colon at age 59. Pathology revealed the presence of a tubular adenoma. She was born in Bauline and now resides in Pouch Cove. Her mother and her maternal grandfather originated from Bauline, and her maternal grandmother originated from Chelsea, Trinity Bay. Her father and paternal grandfather originated from Bauline. Her paternal grandmother originated from Blackhead. The pedigree constructed consists of 44 relatives. She has 7 first degree relatives: 2 children, 3 siblings, and parents. Her mother died of a basal cell carcinoma at age 87. Her father is alive and is age 89. A sister was diagnosed with breast cancer at age 58. There are no other reported cancers in first degree relatives. She has 26 second degree relatives. There were 2 HNPCC cancers reported in her second degree relatives. They included a CRC in a maternal aunt who died at 68 and a lymphoma at 68 in maternal aunt who also had a cervical cancer at 64. A bone cancer was diagnosed in her maternal grandmother who died at 76. No other cancers were reported in the family.

Family members were referred to the Medical Genetics Clinic because several members (maternal cousins) are affected with Bardet-Biedel Syndrome.

Proband number 174 (pedigree 41) is a 57 year old female who was diagnosed, in 1997, with a moderately differentiated cancer of the sigmoid colon at age 52. Pathology revealed 2 small polyps. She was diagnosed with liver metastasis in 2000. She was born in St. John's, where she now resides. The pedigree constructed consists of 96 relatives. She has 16 first degree relatives: 2 children, 12 siblings, and parents. A brother was diagnosed with a skin cancer at age 56 and another brother was diagnosed with a prostate cancer at 63. Her father died at age 56 from throat cancer. No other cancers were reported in first degree relatives. Her mother died at age 87 due to an aneurysm. She has 52 second degree relatives. Two HNPCC cancers are reported in second degree relatives. CRC was diagnosed in a paternal aunt at age 60 and the paternal grandmother at age 50. Leukemia was reported in a maternal aunt at age 82. No other cancers were reported in the family.

MSS and pending IHC (n = 2 families)

Proband number 100 (pedigree 42) is now deceased. He was diagnosed, in 1998, with a well differentiated cancer of the sigmoid colon at age 64. Chart review indicated the presence of 2 tubulovillous polyps and a tubular polyp in 1999. He died 2 years later with liver and lung metastasis. He was also diagnosed with a skin cancer at age 65. A daughter was identified as a proxy. He was born in Port de Grave and resides in Bareneed. His mother and maternal grandfather originated from Bay Roberts. His maternal grandmother originated from Coley's Point. His father and his paternal grandfather originated from Port de Grave. His maternal grandmother originated form

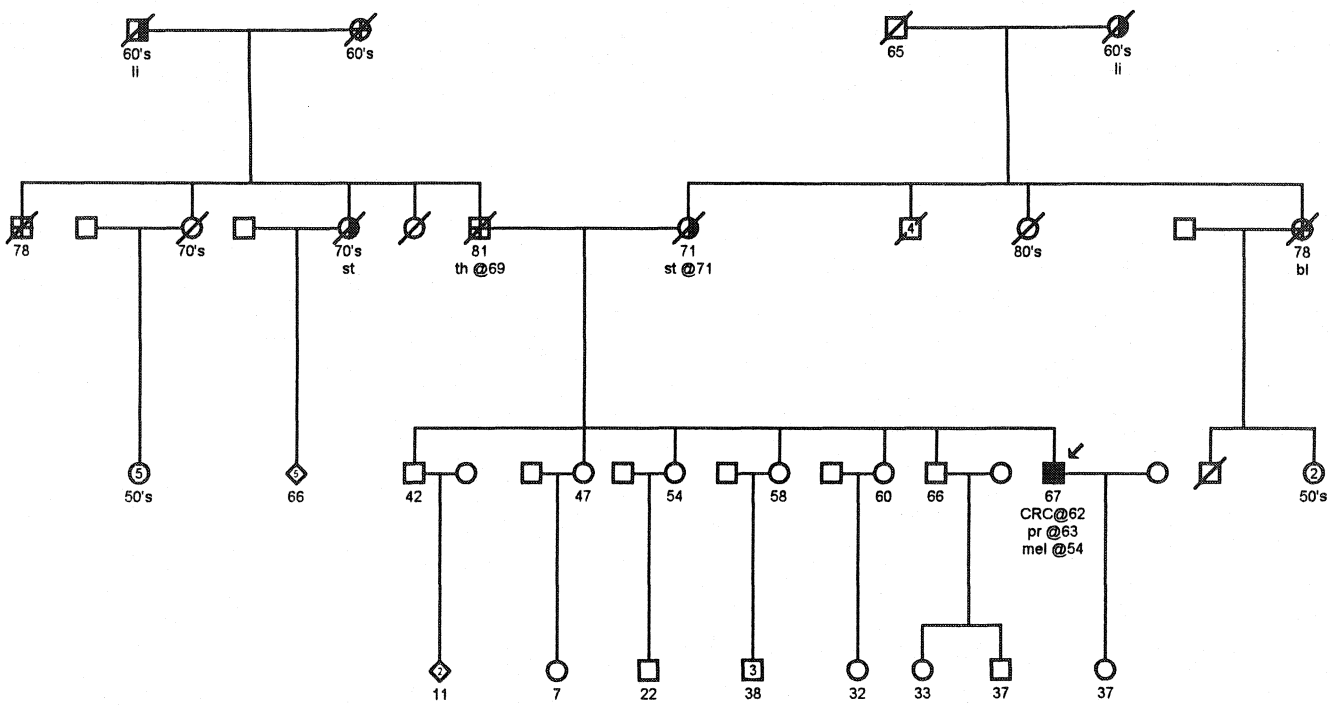
Salmon Cove, South River. The pedigree constructed consists of 77 relatives. He has 10 first degree relatives: 2 children, 6 siblings, and parents. A brother was diagnosed with a HNPCC cancer, CRC, at age 80. His father was diagnosed with leukemia and died at age 61. A brother was diagnosed with prostate cancer at age 61 No other cancers were reported in his first degree relatives. His mother died at age 61 form a stroke. He has 46 second degree relatives. A nephew was diagnosed with skin cancer at age 49. No other cancers were reported in his second degree relatives. There were 5 HNPCC cancers reported in maternal cousins. Two siblings were diagnosed with a CRC, one at age 65 and the other at age 59. Another maternal cousin was diagnosed with a CRC and a stomach cancer at age 53. Two HNPCC cancers were diagnosed in paternal cousins. A CRC was diagnosed at age 60 in one cousin and CRC was reported at age 60 in another cousin. No other cancers were reported in the family. This proband is related to a family previously referred to the Medical genetics program for rod cone dystrophy.

Proband number 137(pedigree 43) is now deceased. He was diagnosed, in 1998, with a well differentiated cancer of the rectosigmoid junction with liver metastasis at age 56. He died the same year. His wife was identified as the proxy. He was born in Manuels. His mother and maternal grandfather originated from Heart's Desire. His maternal grandmother originated from Heart's Content. His father originated from Whitbourne. His paternal grandmother originated from Spaniards Bay and his paternal grandfather originated from Clarke's Beach. The pedigree constructed consisted of 47 relatives. He had 6 first degree relatives: 2 children, 2 siblings, and parents. A HNPCC cancer, stomach (epigastric), was diagnosed in his brother at age 52. His father was diagnosed with prostate cancer and died at age 75. His mother died at age 75 from heart

complications. He had 19 second degree relatives. His paternal grandparents were diagnosed with 2 cancers, type unknown. A maternal aunt was diagnosed with a brain tumour and died at age 80. Two maternal uncles were diagnosed with lung cancers, one at age 57 and the other at age 78. No other cancers were reported in second degree relatives. Two HNPCC cancers were reported in cousins. A paternal cousin was diagnosed with a CRC at age 55. A maternal cousin was diagnosed with a liver cancer, age unknown. No other cancers were reported in this family.

No sample available for Molecular and Pathology analysis (n=1 family)

Proband number 107 (pedigree 44) is a 66 year old male who was diagnosed, in 1998, with a cancer of the descending colon at age 62. He was born in New Harbour, Trinity Bay and now resides in St. John's. His mother originated from Spread Eagle, Trinity Bay. The origin of his maternal grandparents was not given. His father originated from New Harbour and was raised by a "foster" family. The pedigree constructed consists of 55 relatives. He has 10 first degree relatives: 2 children, 6 siblings, and parents. His sister died at age 71 due to metastases from lung cancer. His mother was diagnosed with breast cancer at age 93. No other cancers were reported in first degree relatives. He has 17 second degree relatives. A throat cancer was reported in a maternal aunt at age 40. No other cancers were reported in second degree relatives. Three HNPCC cancers were reported in maternal cousins; a CRC in one cousin (age >50) and CRC and stomach cancer in another cousin at age 59. No other cancers were reported in the family.



Pedigree 34 Intermediate 1-Family 2

MSS
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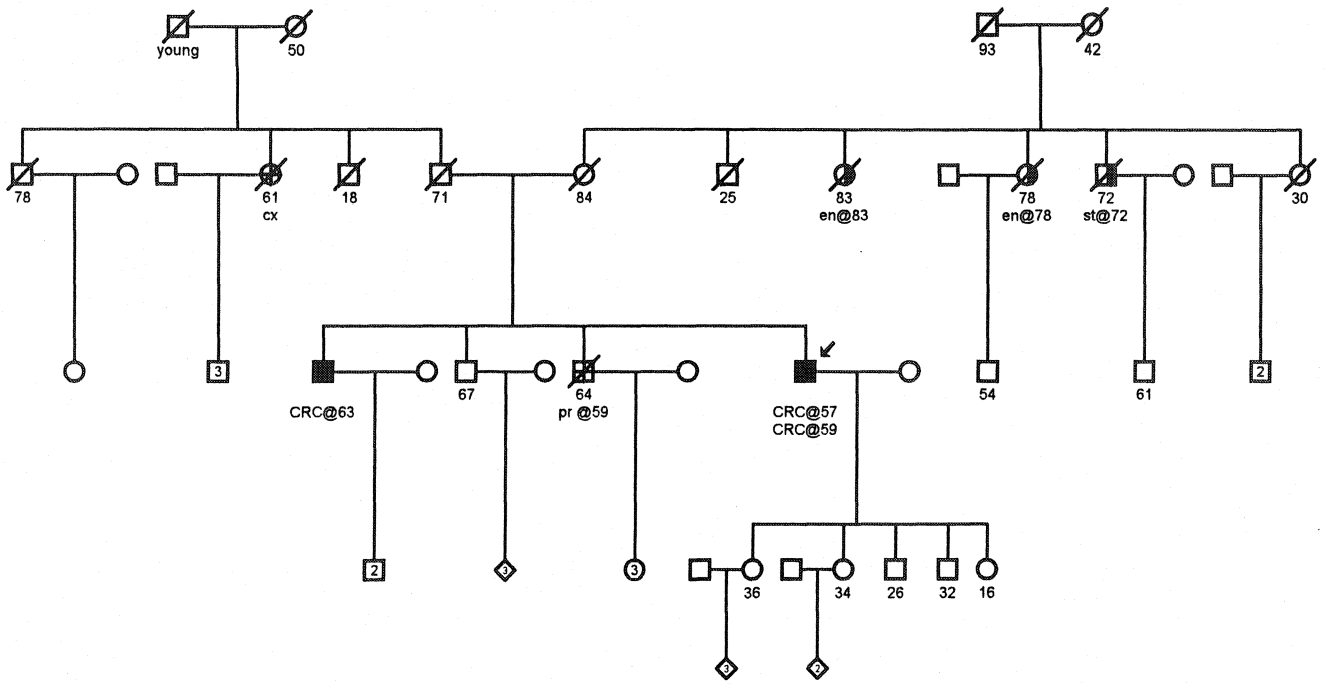

Pedigree 35 Intermediate 1-Family 16

MSS
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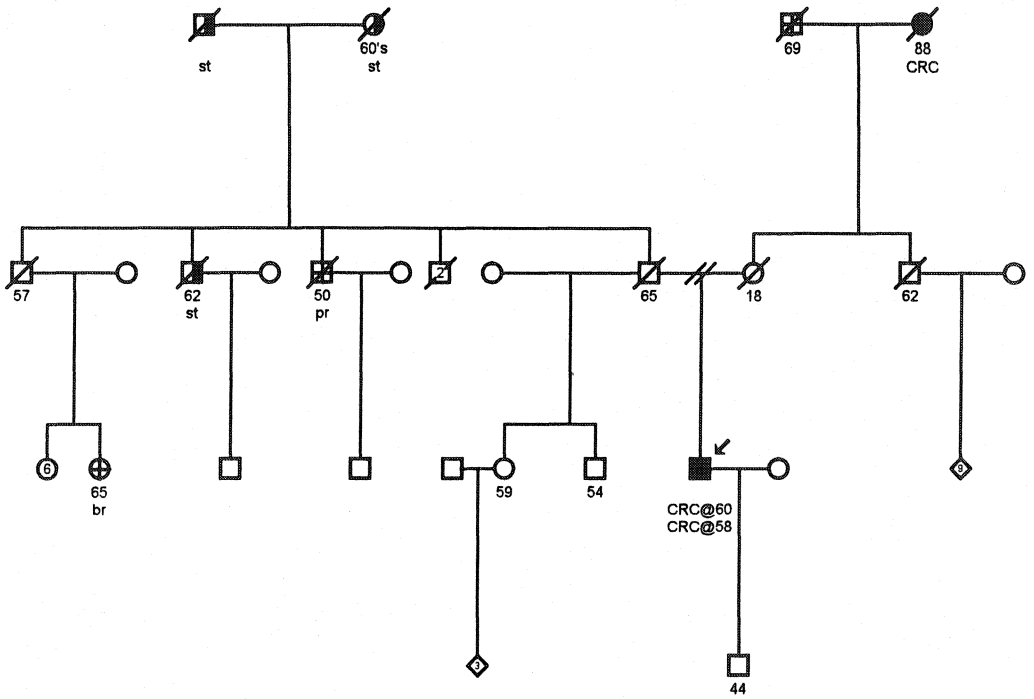

Pedigree 36 **Intermediate 1-Family 21**

MSS
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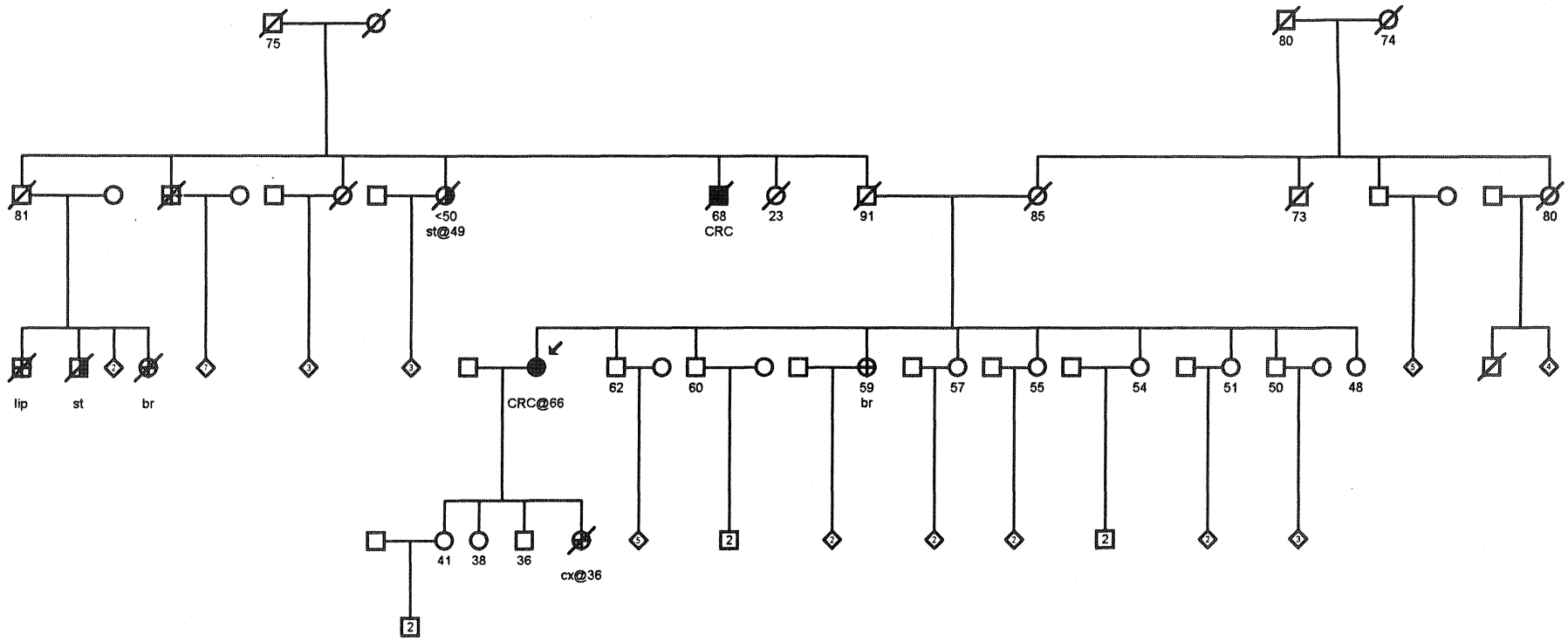

Pedigree 37 Intermediate1-Family 44

MSS
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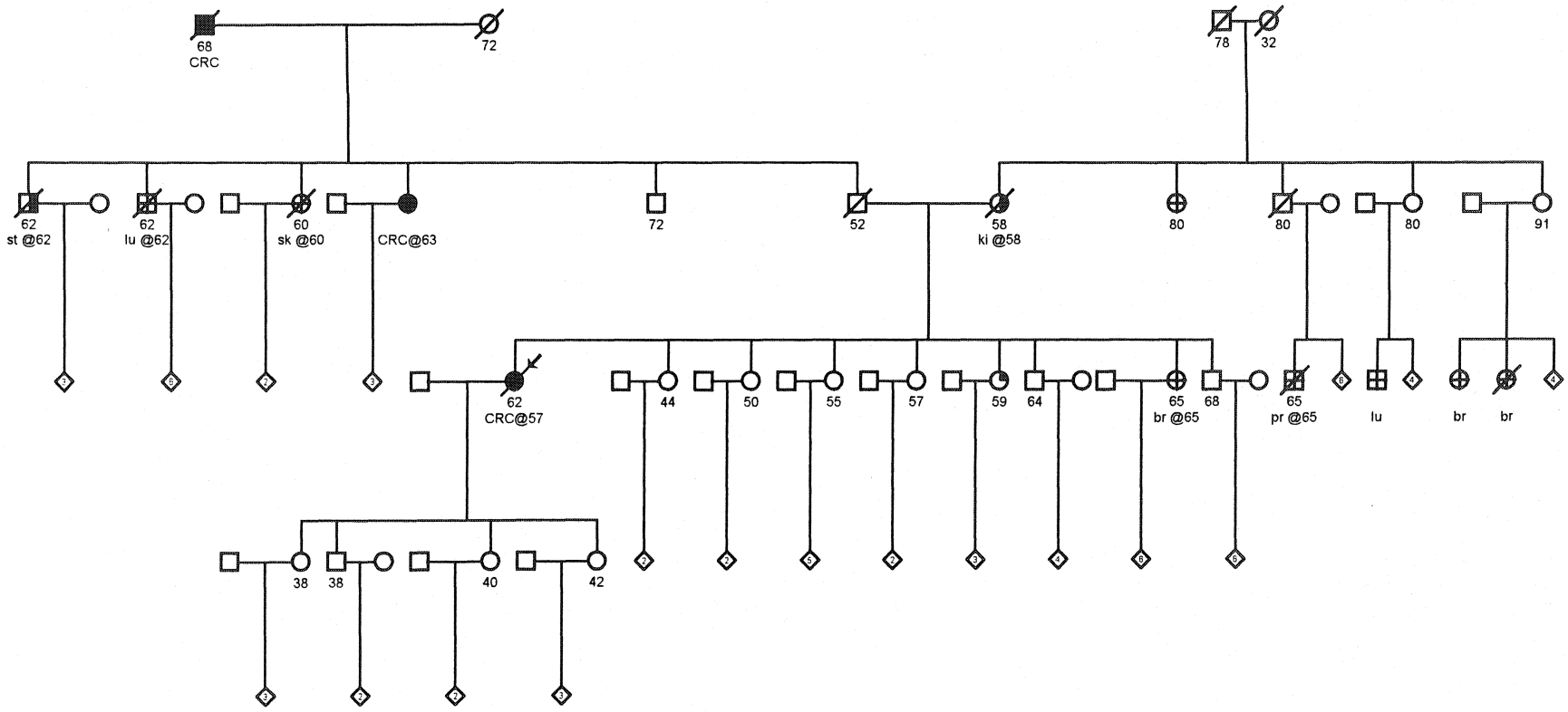
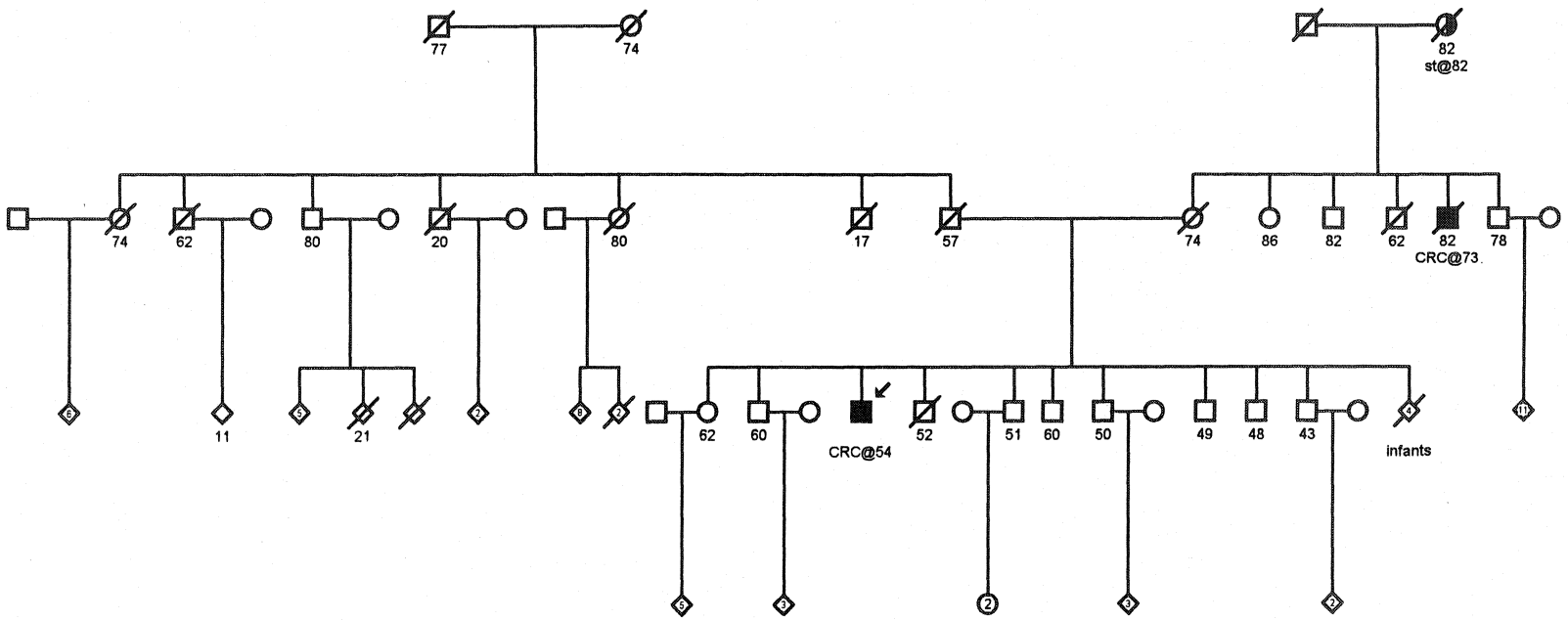


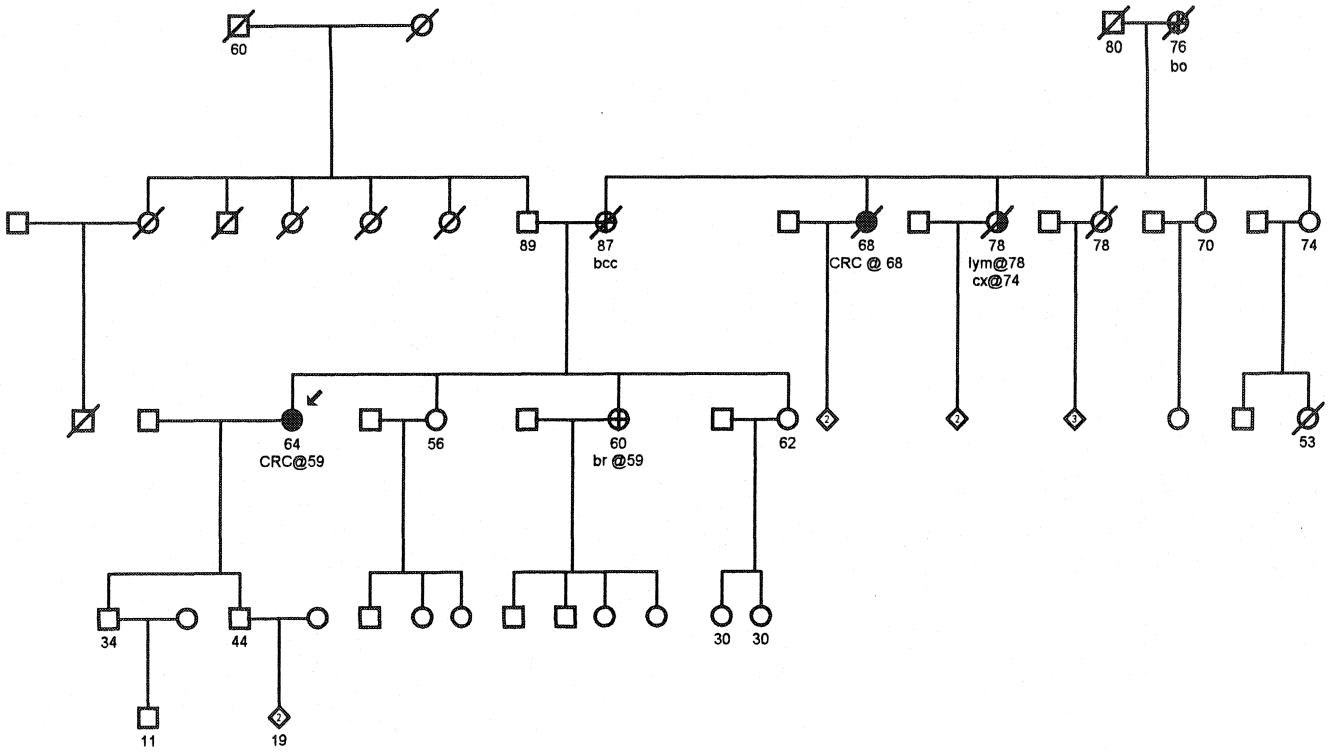
Pedigree 38 **Intermediate-Family 72**

MSS
no loss



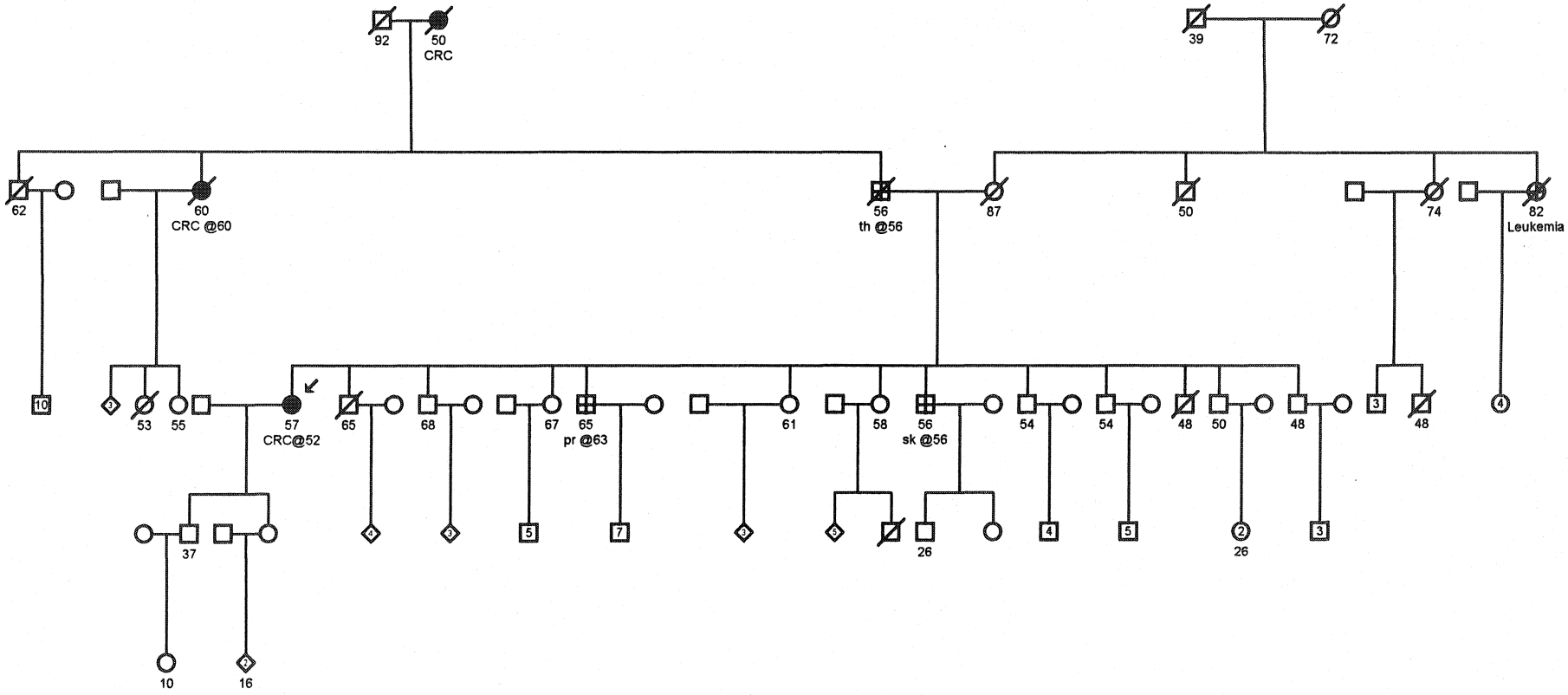
Pedigree 39 **Intermediate 1-Family 126**

MSS
no loss



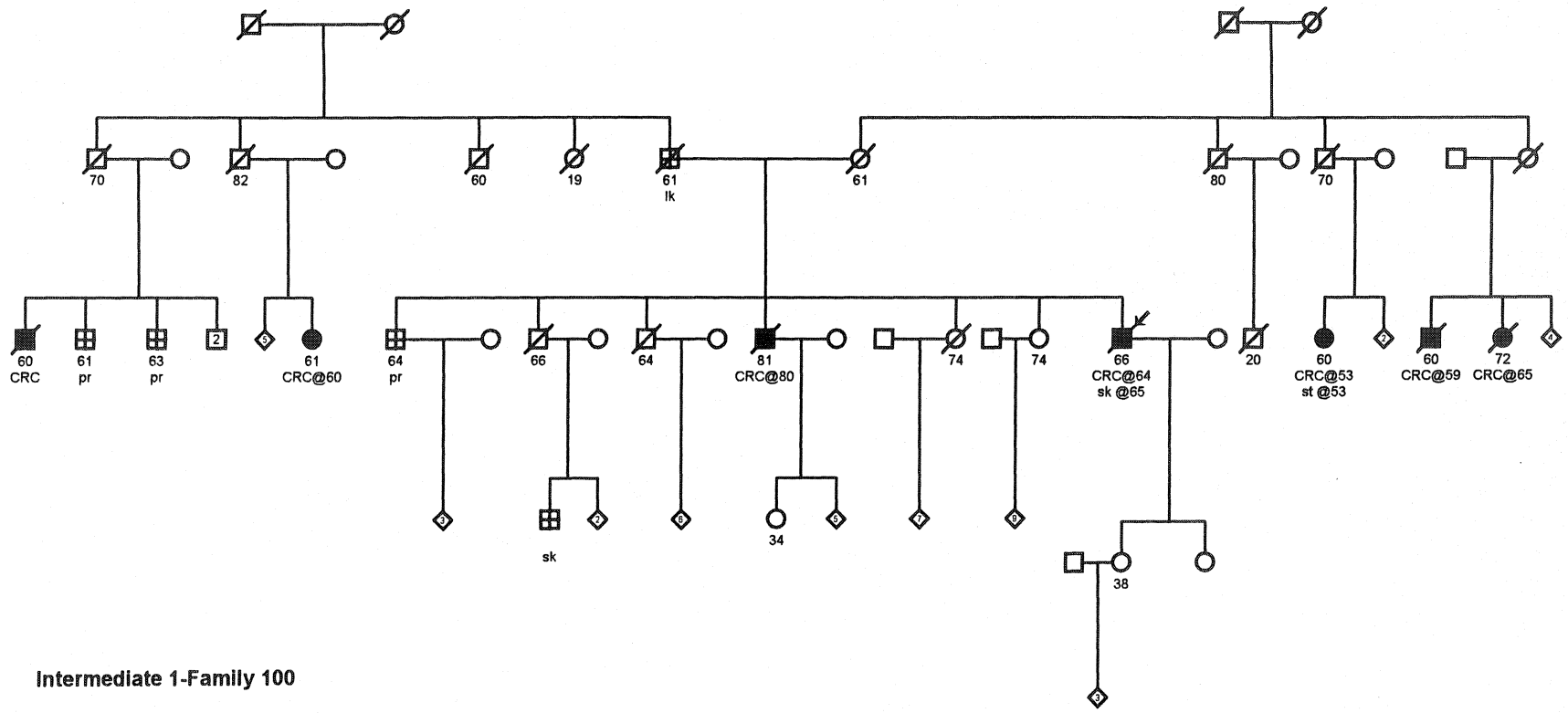
Pedigree 40 **Intermediate 1-Family 112**

MSS
no loss



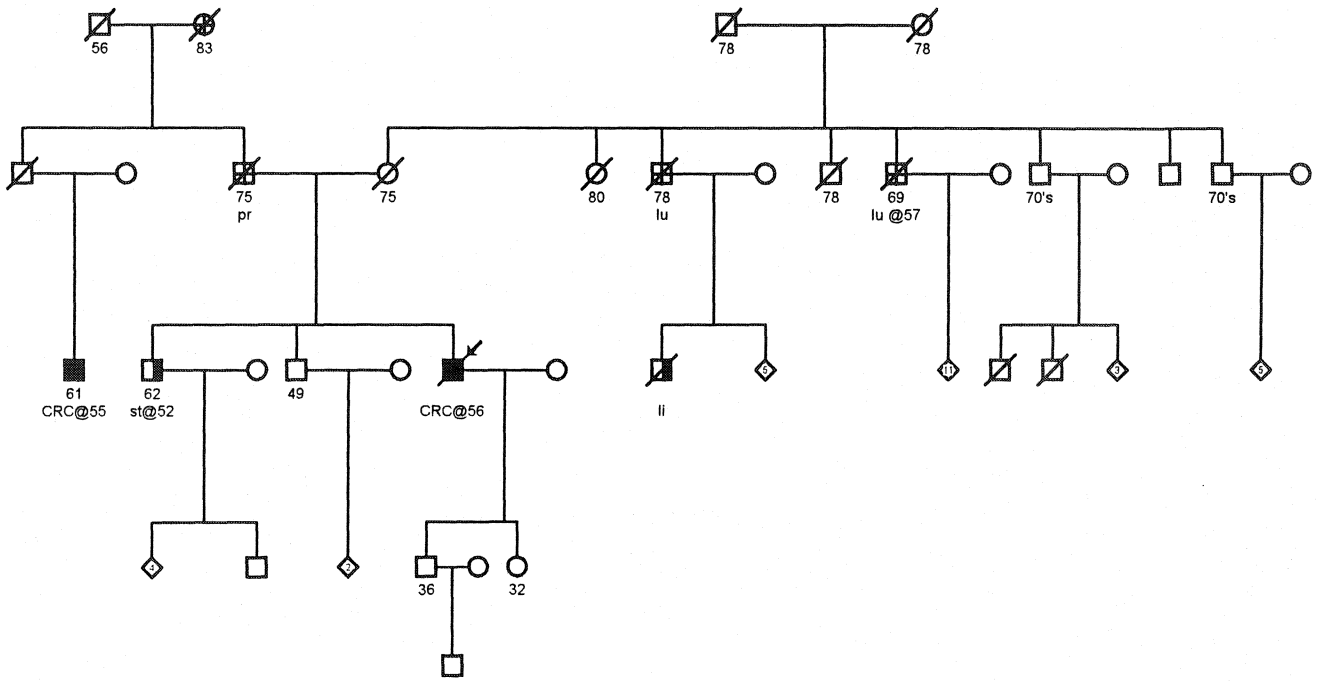
Pedigree 41 Intermediate-Family 174

MSS
no loss



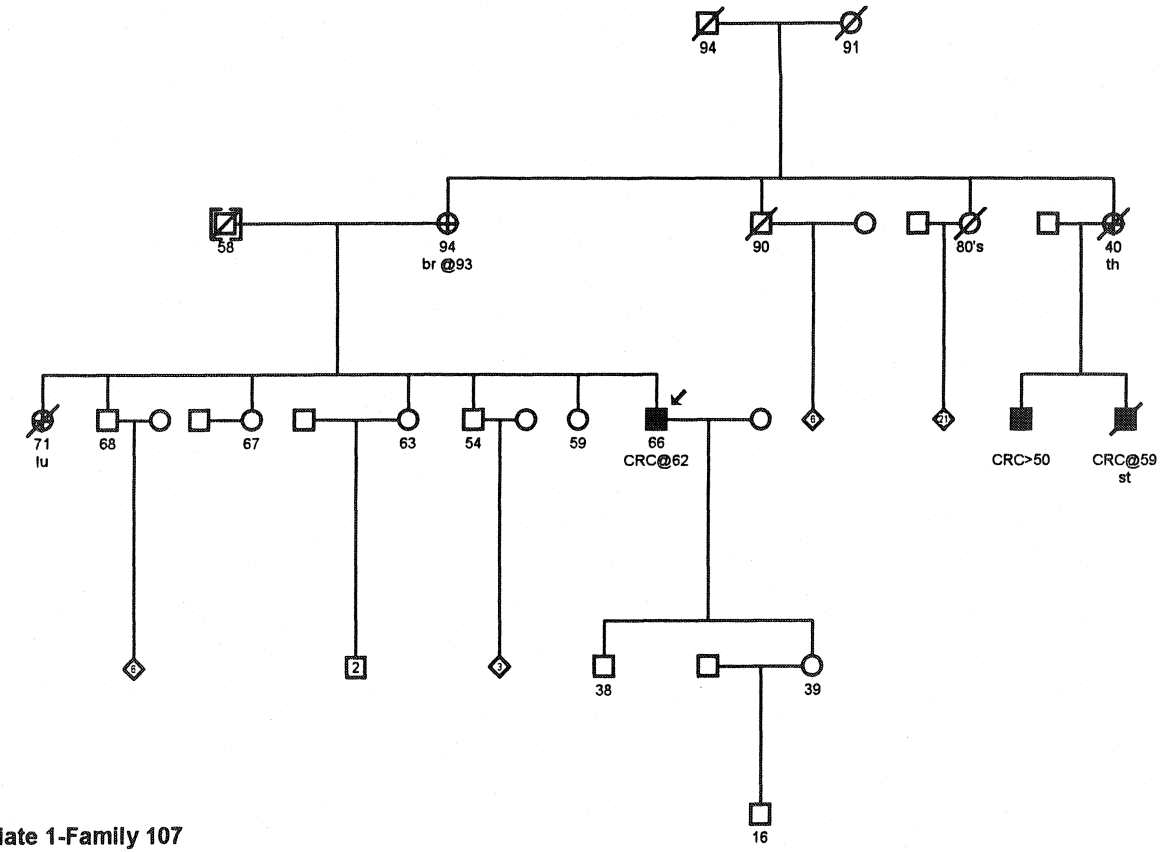
Pedigree 42 Intermediate 1-Family 100

MSS
pending IHC



Pedigree 43 Intermediate 1-Family 137

MSS
pending IHC



Pedigree 44 Intermediate 1-Family 107

No sample

INTERMEDIATE RISK 5 (n =2)

MSS and No loss (n=2 families)

Proband number 6 (pedigree 45) is a 74 year old female who was diagnosed in 1998 with a well differentiated cancer of the transverse colon at age 69. Records revealed that this was her second primary. She was diagnosed in 1996 with a cancer of the ascending colon. She also had a small polyp at the time of surgery. She was born in Carbonear, and now resides in Harbour Grace. Her parents and grandparents were from Carbonear. The pedigree constructed consisted of 41 relatives. She has 8 first degree relatives: 4 children, 2 siblings, and parents. Her father lived to age 61, and he died in 1956 after suffering a severe asthma attack. He was reported to have had a cancer of the prostate. Her mother lived to age 76, and she died in 1970 due to heart trouble. Her two siblings both had a non HNPCC cancer. Her brother died at the age of 68 due to metastasis from a skin cancer. Her sister had a breast cancer, and died at the age of 62 due to complications during a heart surgery. No other cancers were reported in her first degree relatives. She has 23 second degree relatives. Her maternal grandfather died from a HNPCC cancer, stomach cancer, at age 60. She reported a CRC in a paternal aunt at age 50. This aunt lived to be 90. She also reported non HNPCC cancers in 2 paternal aunts and 1 paternal uncle. They were a breast cancer, a prostate cancer and cancer (type unknown). No other cancers were reported in second degree relatives. She also reported a breast cancer at age 53 in a maternal cousin. No other cancers were reported in the family.

Proband number 61 (pedigree 46) is a 72 year old woman who was diagnosed in 1997 with a poorly differentiated cancer of the sigmoid colon at age 66. She was

diagnosed in 1999 with a second primary, a cancer of the rectosigmoid junction. She was born in St. John's where she resides. Her mother and her maternal grandmother originated from Winterton. She was unaware of her maternal grandfather's origin. Her father and her paternal grandfather originated from Whitbourne. She was unaware of the origin of her paternal grandmother. The pedigree constructed consisted of 58 relatives. She has 5 first degree relatives: 1 child, 2 half sibs (different father, previous marriage), and parents. Her mother died at age 83. Her father died at age 73 due to a cerebral haemorrhage. No other cancers were reported in her first degree relatives. She has 14 second degree relatives. Two cancers were reported in her second degree relatives. A breast cancer was reported in a niece at approximately age 50. There was a reported cancer (type unknown) in her maternal grandmother who died in her 60's. No other cancers were reported in the family.

INTERMEDIATE RISK 7- Pathology (n = 4 families)

MSS and No loss (n= 4 families)

Proband number 27(pedigree 47) is a 71 year old female who was diagnosed, in 1997, with a moderately differentiated cancer of the colon at age 66. She was also diagnosed with a breast cancer at age 48. Chart review reveals the presence of 17 hyperplastic and tubular adenomatous polyps in 1998. Follow-up colonoscopies revealed 6 polyps of which 3 were tubular adenomas. Her parents and grandparents originated from Long Pond and Kelligrews, Conception Bay South. The pedigree constructed consists of 66 relatives. She has 9 first degree relatives: 4 children, 3 siblings, and parents. A HNPCC cancer, CRC, and a breast cancer were diagnosed in her mother at age 63. Her brother died at age 26 due to leukemia. No other cancers were reported in

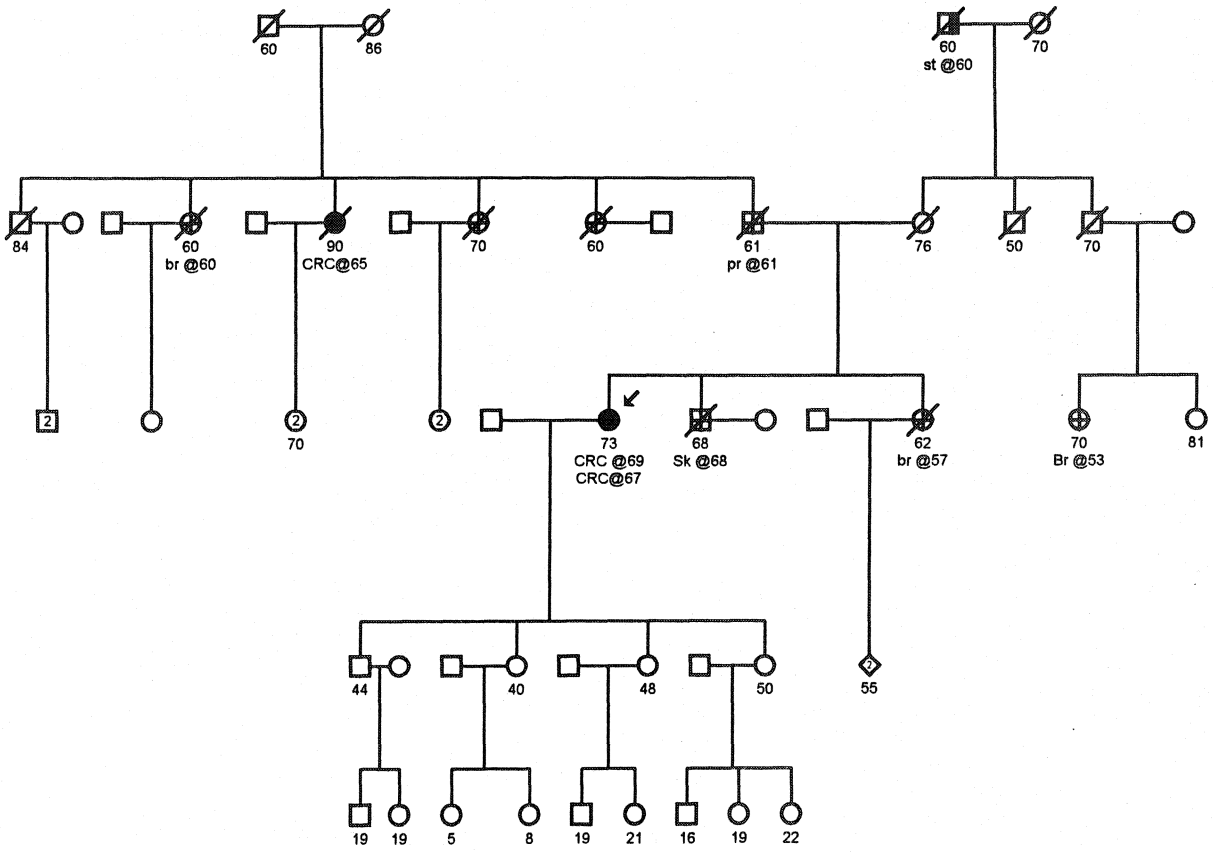
first degree relatives. Multiple polyps are reported in 3 of her children. Her father died due to a stroke at age 76. She has 23 second degree relatives. No cancers were reported in second degree relatives. A HNPCC cancer, CRC, is reported (age unknown) in a paternal cousin. A cancer (type unknown) is reported in another paternal cousin. No other cancers were reported in the family.

Proband number 73 (pedigree 48) is 54 year old man who was diagnosed, in 1998, with a moderately differentiated cancer of the ascending colon at age 49. Pathology revealed the presence of 20 polyps (tubular adenomata). There have been more on follow-up colonoscopies. He was born in Come by Chance, and now resides in Mt. Pearl. His mother originated from Lethbridge, Bonavista Bay. The pedigree constructed consists of 99 relatives. He has 13 first degree relative; 2 children, 9 siblings (6 half siblings), and parents. A HNPCC cancer, esophageal cancer, was reported in a half sister at age 56. A lung cancer was reported in a brother at age 43. No other cancers were reported in first degree relatives. His mother died at age 73 due to a heart attack, and his father died at age 84 due to heart complications. He has 36 second degree relatives. Skin cancer was reported in 2 nieces (maternal) at ages 45 and 54. Another skin cancer was reported in a paternal uncle at age 81. There were no other reported cancers in second degree relatives. A HNPCC cancer, stomach cancer, was reported at age 37 in a paternal cousin. No other cancers were reported in the family.

Proband number 97 (pedigree 49) is a 63 year old male who was diagnosed, in 1998, with a moderately differentiated cancer of the hepatic flexure at age 58. Pathology revealed the presence of a cancer in a polyp and 7 adenomatous polyps. He was recently diagnosed with prostate cancer. He resides in Bishops Cove. His mother's family

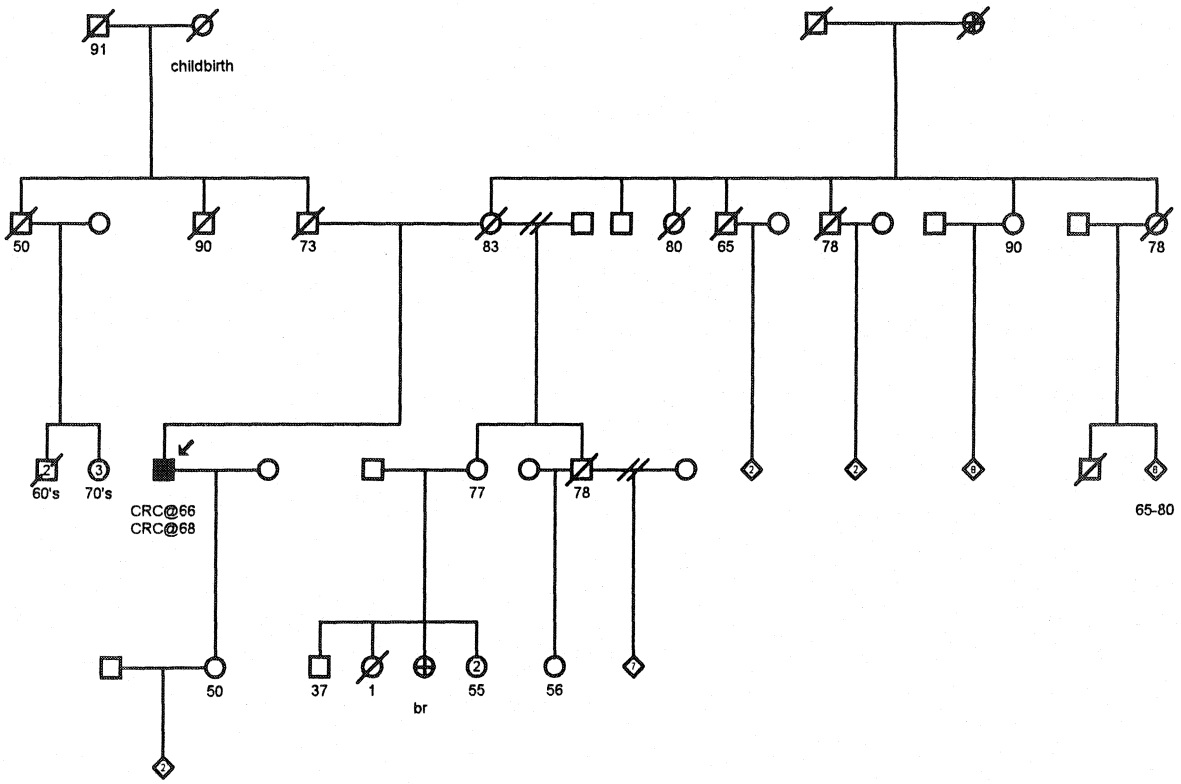
originates from Conception Bay. Information pertaining to his father and paternal family is not available. The pedigree constructed consists of 42 relatives. He has 8 first degree relatives: 4 children, 3 siblings (half, mother remarried), and a mother. His mom died at age 85. His son was diagnosed with HNPCC cancer, lymphoma at age 28. Two brothers were diagnosed with prostate cancer, one at age 49 and the other at age 51. He has 12 second degree relatives. His maternal grandfather was diagnosed with HNPCC cancer, CRC at age 77. A maternal uncle was diagnosed with Hodgkin's at age 40. No other cancers were reported in the family. The proband was previously referred to the Medical genetics program.

Proband number 118 (pedigree 50) is now deceased. He was a male who was diagnosed, in 1997, with a well differentiated cancer of the cecum with lung metastasis. He was diagnosed with a recurrent cancer in 1998. Polyps were reported as multiple villous adenomata with foci of carcinoma in situ. He had a 22 year history of ulcerative colitis. He was born in New York and resided in St. John's. His father and paternal grandparents originated from Italy. His mother and maternal grandparents originated from Pennsylvania. The pedigree constructed consists of 47 relatives. He has 8 first degree relatives; 2 children, 4 siblings, and parents. Breast cancer was reported in his sister at age 49. His mother died at age 59 after she was diagnosed with a choroidal melanoma at age 56. No other cancers were reported in first degree relatives. He has 20 second degree relatives. A HNPCC cancer, endometrial cancer was reported in his maternal grandmother at age 75. No other cancers were reported in the family. The proband was previously referred to the Medical genetic program because several other family members with crohn's and ulcerative colitis.



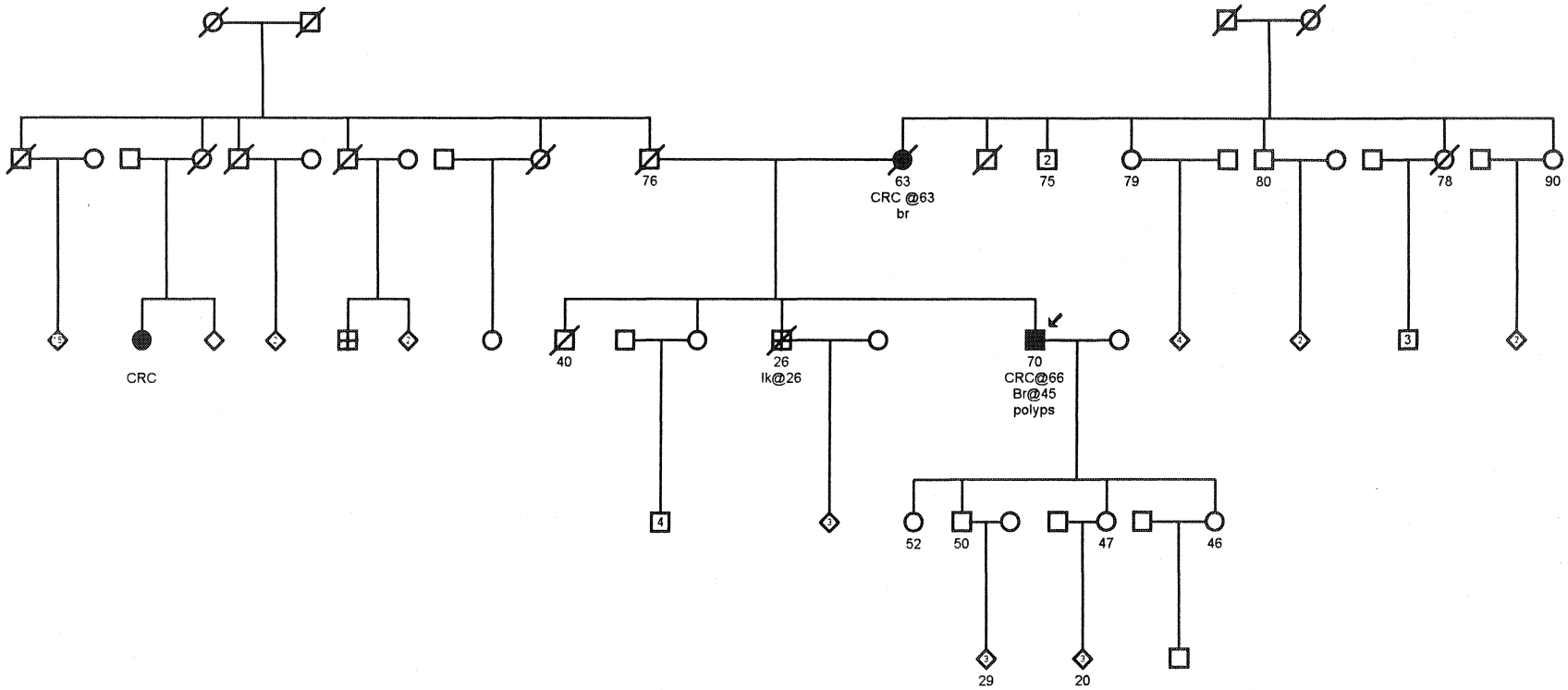
Pedigree 45 Intermediate 5-Family 6

MSS
no loss



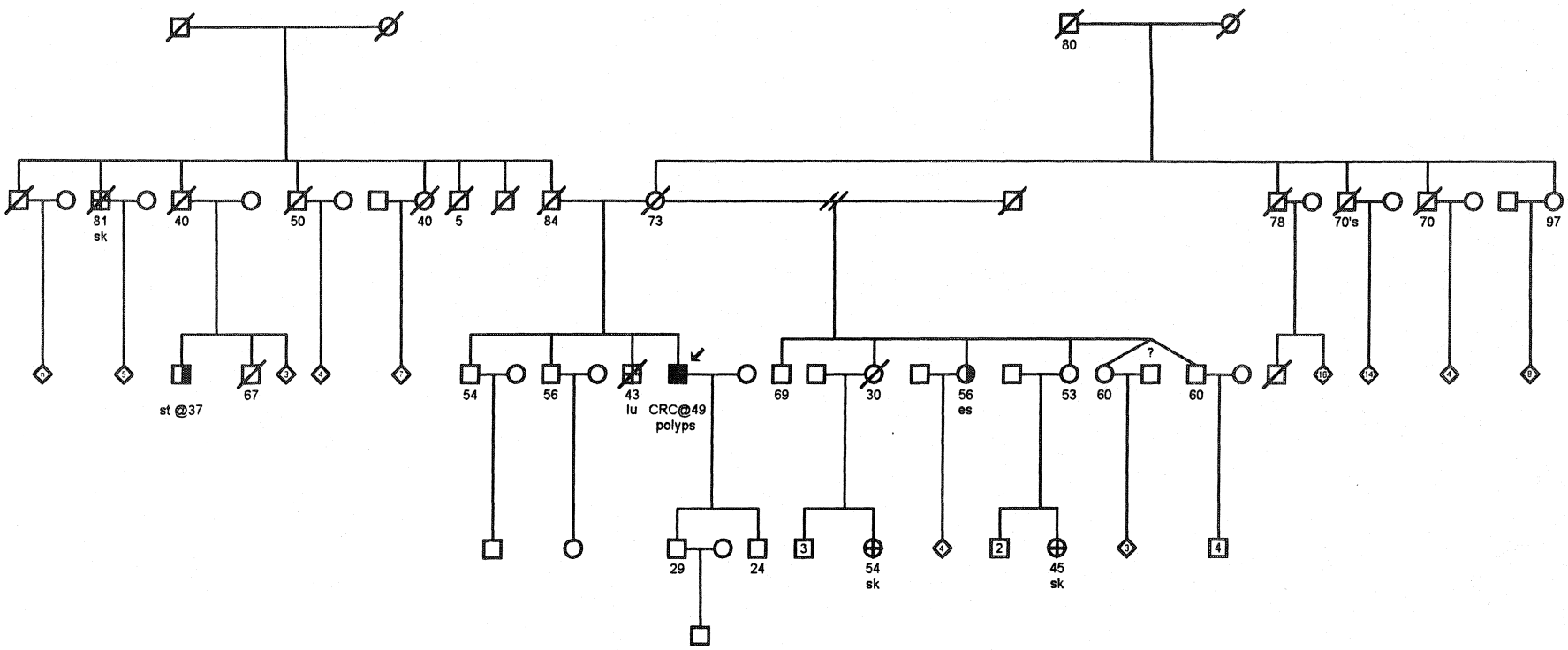
Pedigree 46 Intermediate 5- Family 61

MSS
no loss



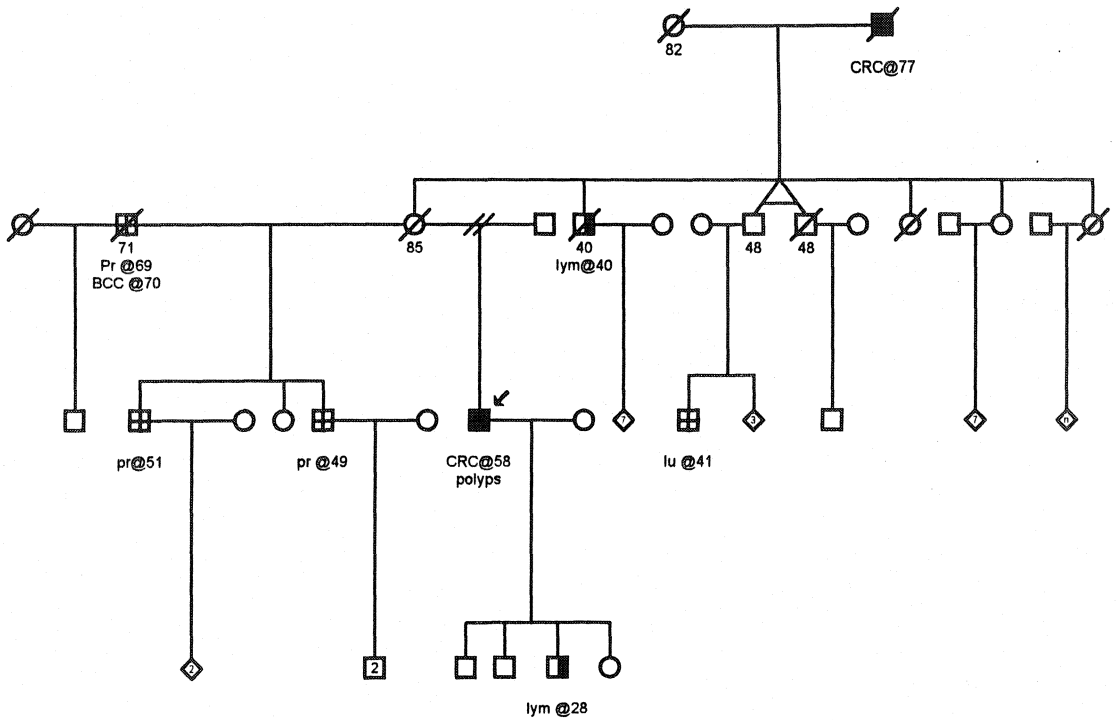
Pedigree 47 Intermediate 7-Family 27

MSS
no loss



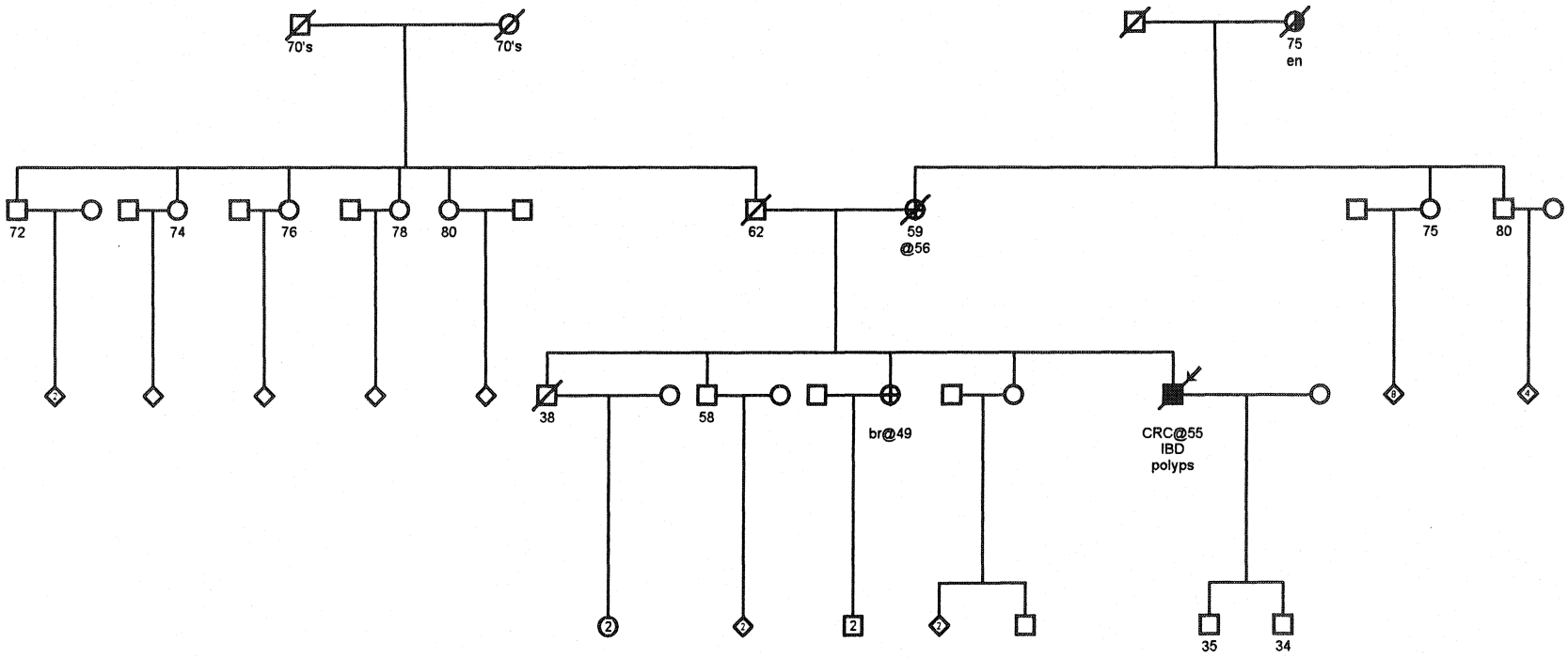
Pedigree 48 Intermediate 7-Family 73

MSS
no loss



Pedigree 49 Intermediate 7-Family 97

MSS
no loss



Pedigree 50 Intermediate 7-Family 118

MSS
no loss

LOW RISK (n=29 families)

Informative (n= 17)

MSS and No loss (15)

Proband number 20(pedigree 51) is now deceased. He was a male who was diagnosed, in 1998, with a poorly differentiated cancer of the transverse colon at age 64. Pathology at the time of surgery revealed the presence of 3 hyperplastic polyps with and a tubular adenoma. He subsequently died at age 65 due to a metastasis to the liver. A review of his medical records was not possible (missing). The proxy identified was a sister. The proband was born in St. Lunaire and lived in St. John's. His mother and maternal grandfather originated from Harry's Harbour and his maternal grandmother originated from Twillingate. His father and paternal grandparents originated from St. Lunaire. The pedigree constructed consists of 68 relatives. The proband had 7 first degree relatives: 5 siblings, and parents. He had no children. His father lived to age 75 and died due to an aneurysm. His mother lived to age 93. Polyps are reported in two of the proband's brothers. No cancers were reported in his first degree relatives. He had 27 second degree relatives. A HNPCC cancer, lymphoma was reported in a paternal aunt at age 78 and a leukemia was reported in a paternal cousin at age 29. No other cancers were reported in the family.

Proband number 54 (pedigree 52) is a 72 year old woman who was diagnosed in 1997 with a moderately differentiated cancer of the descending colon at age 66. One polyp was also reported. She was also diagnosed with a basal cell carcinoma on the face. She was born in Botwood, where she now resides. Information about the parents and

grandparents demographics was not given. The pedigree drawn consists of 37 relatives. There are 10 first degree relatives: 2 children, 6 siblings, and parents. Her mother died at 72 due to CVA. Her father died at age 82 due to complications of Alzheimer's. There is a bone cancer reported in a sibling at age 72. No other cancers were reported in the first degree relatives. There are 34 second degree relatives. A HNPCC cancer, kidney cancer with metastasis to the brain was reported in a nephew at age 50. A breast cancer was reported in a niece at age 47. No other cancers were reported in the family.

Proband number 56 (pedigree 53) is a 45 year old woman who was diagnosed, in 1998, with a moderately differentiated cancer of the rectum at age 42. A sessile polyp is also reported. She was born in New Melbourne and now resides in St. John's. Her mother and maternal grandparents originated from Whiteway, Trinity Bay. Her father and her paternal grandparents originated from New Melbourne, Trinity Bay. The pedigree constructed consists of 66 relatives. There are 8 first degree relatives: 1 child, 5 siblings, and parents. Both of her parents are still alive, her mother is 78, and her father is 79. There are no cancers reported in the first degree relatives. There are 23 second degree relatives. There are 4 HNPCC cancers, 3 CRC and an ovarian cancer, all primaries, in one paternal aunt at age 59. There were 2 cancers (type unknown) reported in 2 paternal uncles. A HNPCC cancer, CRC was reported in a maternal aunt in her early 50's. No other cancers were reported in the family.

Proband number 82 (pedigree 54) is a 58 year old male who was diagnosed, in 1998, with a moderately differentiated cancer of the rectum at age 55. He was born in St. John's and now resides in Portugal Cove. His mother and his maternal grandfather originated from New Melbourne. His Maternal grandmother originated from Dunfield,

Trinity Bay. His father originated from New York City. Because his father originated from New York City, he was unaware of his paternal family history. The pedigree constructed consists of 34 relatives. He has 8 first degree relatives: 2 children, 4 siblings, and parents. His mother died from diabetes complications at age 65, and his father died from MI at age 80. No cancers were reported in his first degree relatives. He has 15 second degree relatives. No cancers were reported in the family.

Proband number 84 (pedigree 55) is a 52 year old male who was diagnosed, in 1998, with a moderately differentiated cancer of the rectum at age 45. Pathology revealed the presence of a sessile polyp. He was born in St. John's, and he now resides in Mt. Pearl. His mother and his maternal grandfather originated from Colinet Island, St. Mary's Bay. His father and his parents originated from St. John's. The pedigree constructed consists of 61 relatives. He has 11 first degree relatives: 2 children, 2 siblings, and parents. His mother is 73 years old. His father died of metastases to the lung from a prostate cancer at age 72. No other cancers are reported in first degree relatives. He has 35 second degree relatives. A HNPCC cancer, CRC was reported in his paternal grandfather who died at age 75. A cancer of the bone was reported in a paternal uncle who died at age 65, and a cancer (type unknown) was reported in another paternal uncle. He reported leukemia in his maternal grandfather who died at age 78 and a cancer (type unknown) in a maternal uncle, who died at age 70. No other cancers were reported in the family.

Proband number 121(pedigree 56) is a 44 year old female who was diagnosed, in 1997, with a poorly differentiated cancer of the cecum at age 37. The proband was born in St. John's where she now resides in St. John's. Her maternal family came from Old

Perlican. The origins of her paternal family were unknown. The pedigree consists of 47 relatives. She has 14 first degree relatives: 1 child, 11 siblings, and parents. Her mother died at age 71 from heart complications. A sister is also deceased from heart complications. Polyps are reported in 2 siblings. No cancers were reported in first degree relatives. She has 35 second degree relatives. A HNPCC cancer, stomach cancer was reported in a maternal uncle in his 70's. A breast cancer is reported in a maternal aunt at age 64, a lung cancer is reported in a maternal uncle at age 72, and a prostate cancer (age unknown) in a maternal uncle. No other cancers were reported in the family.

Proband number 125 (pedigree 57) is a 73 year old male who was diagnosed, in 1997, with a moderately differentiated cancer of the rectum at age 68. Chart review indicates an adenoma and hyperplastic polyps. He was born in LongPond where he now resides. His mother originated from Paradise. His maternal grandparents originated from Island Cove. His father and paternal grandfather originated from Longpond. His paternal grandmother originated from Port de Grave. The pedigree constructed consists of 37 relatives. He has 7 first degree relatives: 3 children, 2 siblings, and parents. His mother is alive and is age 97. His father died of heart complications at age 62. No cancers were in his first degree relatives. He has 24 second degree relatives. A HNPCC cancer, stomach cancer was reported in a maternal uncle who died in his 70's. A breast cancer was reported in his maternal grandmother at age 81 and a cancer (unknown type) in a maternal aunt who died at 68. No other cancers were reported in the family.

Proband number 161(pedigree 58) is a 62 year old male who was diagnosed, in 1997, with a moderately differentiated cancer of the cecum at age 57. He was born in Hants Harbour. His mother and maternal grandfather originated from Hants Harbour.

His maternal grandmother originated from Hearts Content. His father and paternal grandparents originated from Hants Harbour. The pedigree constructed consists of 45 relatives. He has 11 first degree relatives: 3 children, 6 siblings, and parents. His mother is still alive and is age 86. His father died from heart complications at age 76. A son was diagnosed with chrons at age 20. No cancers were reported in first degree relatives. He has 19 second degree relatives. No cancers were reported in second degree relatives. There is a cancer (unknown type) reported in a maternal cousin who died at age 49. No other cancers were reported in the family.

Proband number 166 (pedigree 59) is a 62 year old male who was diagnosed with a cancer of the rectum at age 58. He was born in Little Harbour West, Placentia Bay. His mother and his maternal grandparents originated from Little Harbour West, Placentia. His father and paternal grandfather originated from Baie de Lou. His paternal grandfather originated from Oderin. The pedigree constructed consists of 91 relatives. He has 14 first degree relatives, 4 children, 8 siblings, and parents. His mother died from heart complications at age 80. His father died from heart complications at age 82, and he also had a skin cancer diagnosed at 58. A sister died of a pancreatic cancer at age 49. A brother was diagnosed with a skin cancer, he is now 52. He has 40 second degree relatives. There were 3 cancers reported in the second degree relatives. They included a breast cancer in a paternal aunt who died at age 80, a lung cancer in a paternal uncle who died at 49, and a skin cancer in a paternal uncle who died at age 50. No other cancers were reported in the second degree relatives. There is a HNPCC cancer, stomach cancer and a prostate cancer reported in 2 maternal cousins. No other cancers were reported in the family.

Proband number 22 (pedigree 60) is a 62 year old male who was diagnosed, in 1998, with a moderately differentiated cancer of the sigmoid at age 58. Pathology at the time revealed the presence of a small hyperplastic polyp and a tubular adenoma. Chemotherapy was required. His cancer reoccurred in 2000. He is currently undergoing treatment for metastases to the liver and lungs. He was born in Woodstock, Ontario. He now resides in St. John's. His mother and his maternal grandmother originated from Ontario. His maternal grandfather originated from Surrey, England. His father and his paternal grandparents originated from Ontario. The pedigree constructed has 28 relatives. He has 5 first degree relatives: 3 siblings, and parents. He has no children. He reported that his father had a total colectomy at age 40 because of colonic polyposis and died at the age of 67 due to heart complications. Further investigation of his father's records is needed to rule out FAP or AFAP in the proband's family. His mother lived to age 83. No other cancers were reported in his first degree relatives. He has 14 second degree relatives. A paternal aunt had a bone cancer and died at 55. No other cancers were reported in the family.

Proband number 24 (pedigree 61) is a 56 year old man who was diagnosed, in 1998, with a moderately differentiated cancer of the sigmoid at age 52. He was born in Freshwater, Carbonear. He now resides in Norman's Cove. His mother and maternal grandfather originated from Carbonear. His Maternal grandmother originated from Western Bay, CB. His father and his paternal grandfather originated from Carbonear. His paternal grandmother originated from Small Point, Conception Bay. The pedigree constructed consists of 50 relatives. He has 9 first degree relatives: 2 children, 5 siblings, and parents. His father died at age 59 of a HNPCC cancer, stomach cancer, for which he

had no surgery. His father also had a benign brain tumour diagnosed at age 51. His mother is still alive and is 87 years old. A brother had a pre-cancerous mole removed at age 59. Polyps are reported in 2 other siblings. No other cancers were reported in his first degree relatives. He has 14 second degree relatives. A lung cancer is reported in his maternal grandfather. No other cancers were reported in the family.

Proband number 41 (pedigree 62) is a 72 year old male who was diagnosed, in 1997, with a well differentiated cancer of the sigmoid colon at age 67. He now resides in Dunville. He was born in Burin. His Mother and maternal grandparents originated form Marystown. His father originated from Burin. His Paternal grandmother originated from Paradise. His Paternal grandfather originated from Marystown. The pedigree constructed consists of 129 relatives. He has 17 1st degree relatives: 6 children, 9 siblings, and 2 parents. His mother died an accidental death at age 58. His father died at age 54 due to a ruptured appendix. There were 2 cancers reported in his siblings. They included a melanoma at age 55 and a prostate cancer (age not given). All of his children were scoped. He has 22 second degree relatives. No other cancers were reported in his second degree relatives. There were 2 reported cancers in maternal cousins. They included a prostate cancer (age not given), and a brain cancer where the affected was dead by age 60. A Prostate cancer was reported in a paternal cousin who died at age 89. No other cancers were reported in the family.

Proband number 81 (pedigree 63) is a 64 year old male who was diagnosed, in 1998, with a moderately differentiated cancer of the cecum at age 61. Pathology at the time also revealed the presence of a hyperplastic polyp. Chart review indicated the

presence of a small tubulovillous polyp in 1999, 2 small tubular adenomas in 2000, and a small tubular adenoma in 2002. He was born in St. John's and now resides in Mt. Pearl. His mother and maternal grandfather originated from St. John's. His maternal grandmother originated from Harbour Main. His father and his parents originated from St. John's. The pedigree constructed consists of 66 relatives. He has 10 first degree relatives: 5 children, 3 siblings, and parents. His mother died from diverticulitis at age 69, and his father died of lung cancer at age 49. No other cancers were reported in his first degree relatives. He has 17 second degree relatives. He reported 3 cancers in his second degree relatives: a prostate cancer in a maternal uncle, a throat cancer in a maternal aunt, and a breast cancer in a paternal aunt. He reported 2 other cancers in maternal cousins. They included a lung cancer at age 64 and a breast cancer at age 59. No other cancers were reported in the family.

Proband number 92(pedigree 64) is a 62 year old female who was diagnosed, in 1997, with a moderately differentiated cancer of the sigmoid colon at age 57. Pathology revealed the presence of a polyp. She was born in Upper Island Cove, Conception Bay and now resides in St. John's. Her parents and her grandparents originated from Upper Island Cove. The pedigree constructed consists of 66 relatives. She has 10 first degree relatives: 5 children, 3 siblings, and parents. Her mother died of childbirth complications at age 29. Her father died of heart complications at age 57. No cancers were reported in her first degree relatives. She has 33 second degree relatives. Two HNPCC cancers, CRC were reported in a maternal uncle who died at age 80, and in a paternal aunt who died at age 80. A cancer (type unknown) was reported in another paternal aunt who died at age 70. No other cancers were reported in the family.

Proband number 115 (pedigree 65) is a 60 year old female who was diagnosed, in 1998, with a moderately differentiated cancer of the rectosigmoid junction at age 55. Chart review indicated the removal of three benign polyps in 1999. She was born in St. John's and now resides in Paradise. Her mother originated from Wesleyville. Her maternal grandmother originated from Fogo. She did not know where her maternal grandfather originated. Her father and her paternal grandfather originated from Winterton, Trinity Bay. She did not know where her paternal grandmother originated. The pedigree constructed consists of 35 relatives. She has 3 first degree relatives: 1 sibling and parents. Her mother died from diabetes complications at age 78. Her father died from natural causes at age 92. No cancers were reported in her first degree relatives. She has 18 second degree relatives. A HNPCC cancer, CRC was reported in a maternal aunt at age 55. A breast cancer was reported in a paternal cousin in her 60's. No other cancers were reported in the family. During consultation with Dr. Green it was noted that her maternal grandfather is a member of a previously identified "family C". The cancers reported in her family appear to be unrelated. Her blood sample (not yet collected) will be sent for mutation testing.

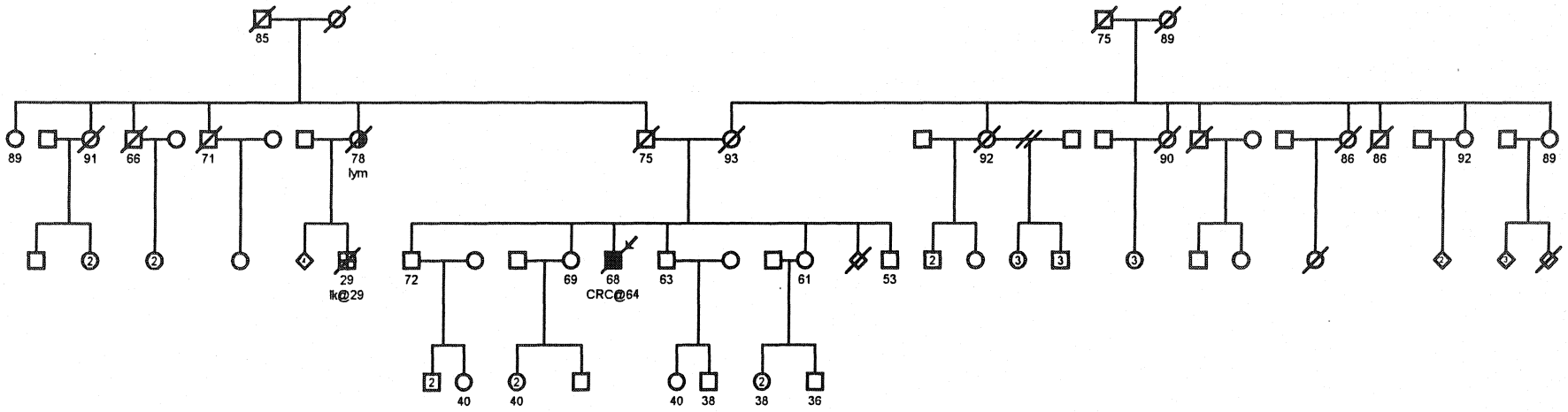
Pending Laboratory results (n=2)

Proband number 116 (pedigree 66) is now deceased. She was diagnosed, in 1997, with a poorly differentiated cancer of the rectosigmoid junction with liver metastasis at age 58. She subsequently died at age 59. Chart review indicated the presence of multiple benign hyperplastic polyps. Her daughter and her father were identified as the proxy. The proband was born in Woody Point, Bonne Bay and she resides in Holyrood. Her

mother and her maternal grandfather originated from Woody Point. Her maternal grandmother originated from Labrador, Lanse aux Clair. Her father originated from Woody Point, Bonavista Bay. Her paternal grandfather originated from Troix Rivieres, Quebec. The origin of her paternal grandmother was not known. The pedigree constructed consists of 42 relatives. She has 17 first degree relatives; 5 children, 10 sibling, and parents. Breast cancers were reported her mother at age 76 and in 2 sisters at ages 44 and 48. No other cancers were reported in first degree relatives. She has 33 second degree relatives. Breast cancer was reported in a maternal aunt at age 31 and the maternal grandmother at age 82. A breast cancer was also reported in a paternal aunt in her 40's. No other cancers were reported in second degree relatives. Three breast cancers were reported in 3 maternal cousins (siblings) at ages 49, 59, and age unknown. . Her husband was also diagnosed with a colorectal cancer at age 58. There are no other reported cancers in the family. This family was previously referred to the Medical Genetics Clinic because of a significant family history of breast cancer

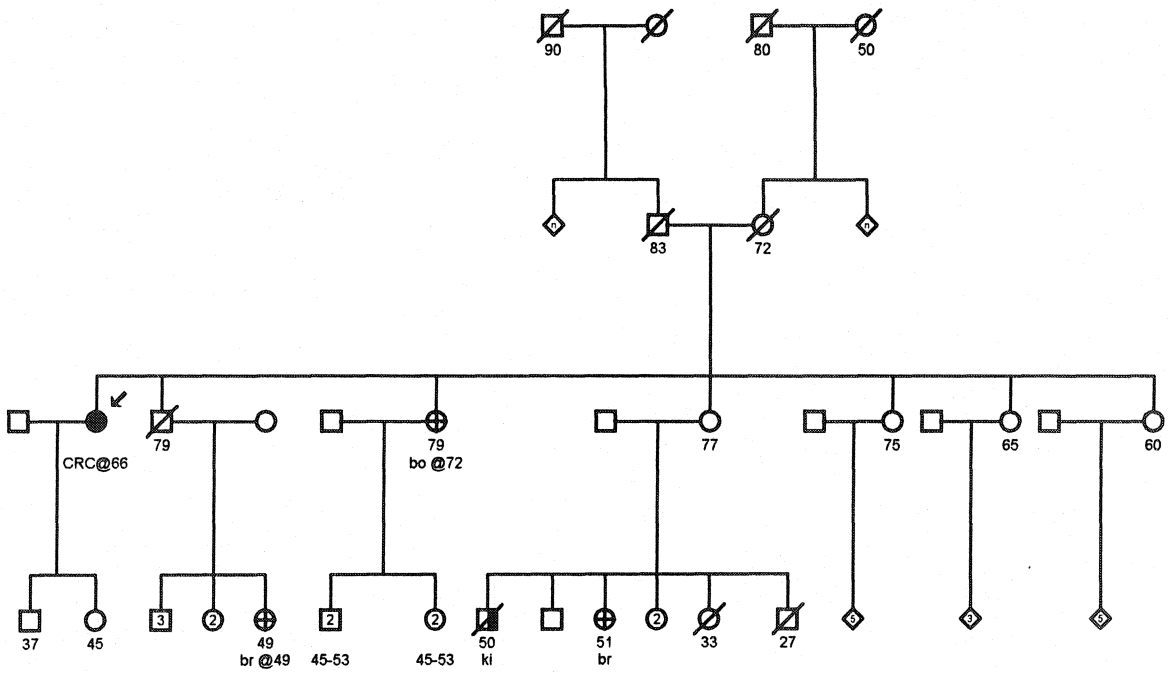
Proband number 145(pedigree 67) is a 60 year old male who was diagnosed, in 1997, with a moderately differentiated cancer of the rectum at age 56. He was born in St. John's where he now resides. His parents and grandparents originated from St. John's. The pedigree constructed consists of 39 relatives. He has 13 first degree relatives: 4 children, 7 siblings, and parents. His mother died of heart complications at age 79. His father died from complications of surgery at age 81. He has no reported cancers in his first degree relatives. He has 23 second degree relatives. There were 2 reported cancers in his second degree relatives: a pancreatic cancer in a paternal uncle who died at age 72

and a breast cancer in a paternal uncle who died at age 65. No other cancers were reported in the family.



Pedigree 51 **Low-Family 20**

MSS
no loss



Pedigree 52 **Low-Family 54**

MSS
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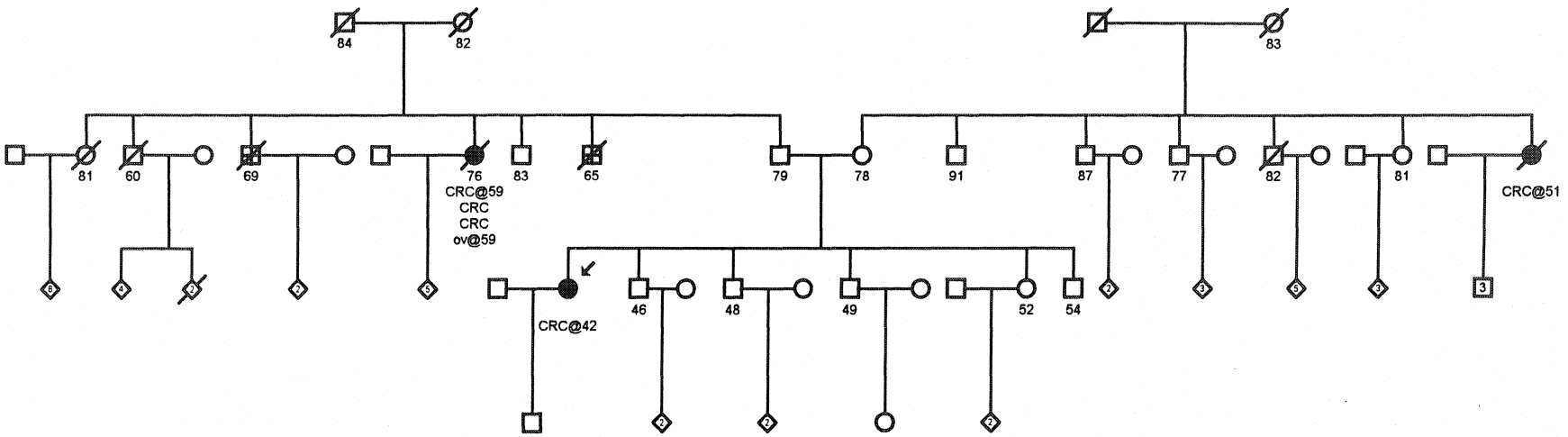
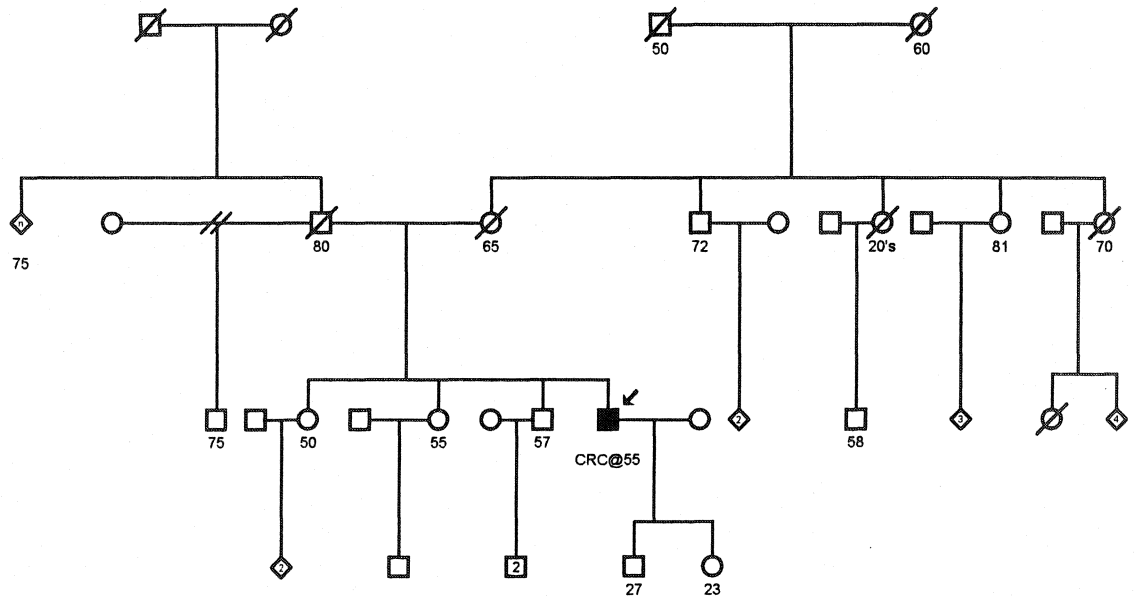


Pedigree 53 **Low-Family 56**

MSS
no loss



Pedigree 54 **Low-Family 82**

MSS
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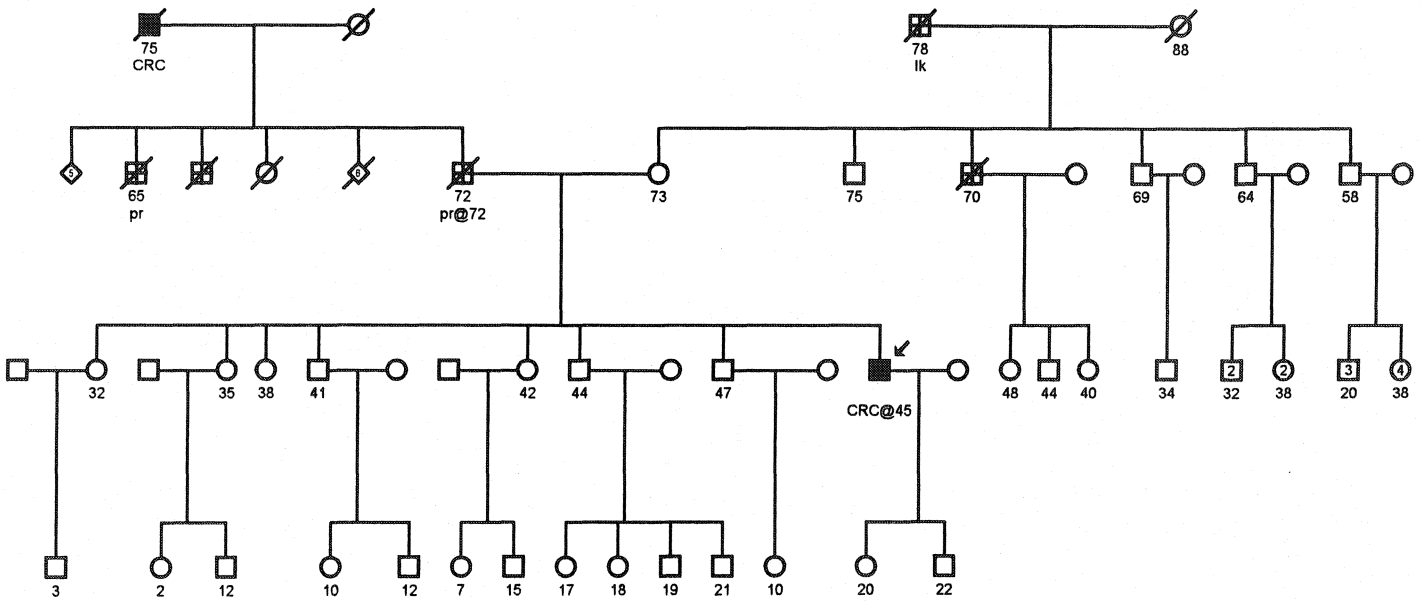
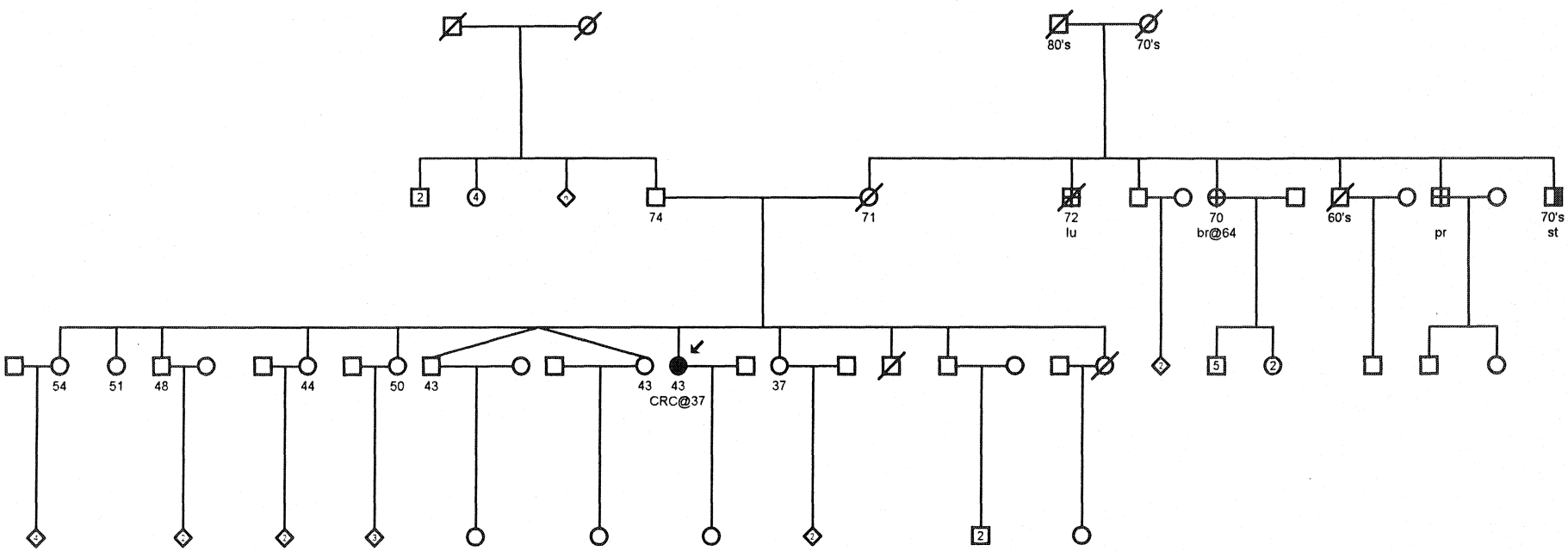


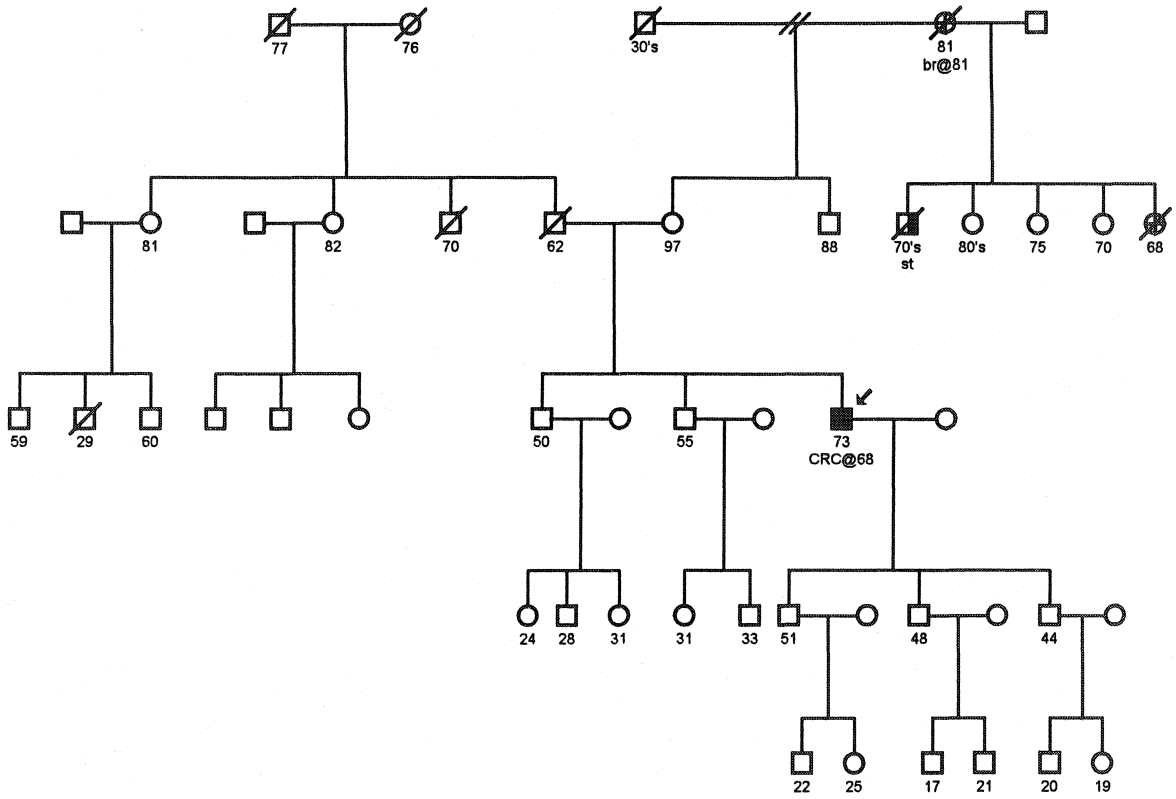
Pedigree 55 Low-Family 84

MSS
no loss



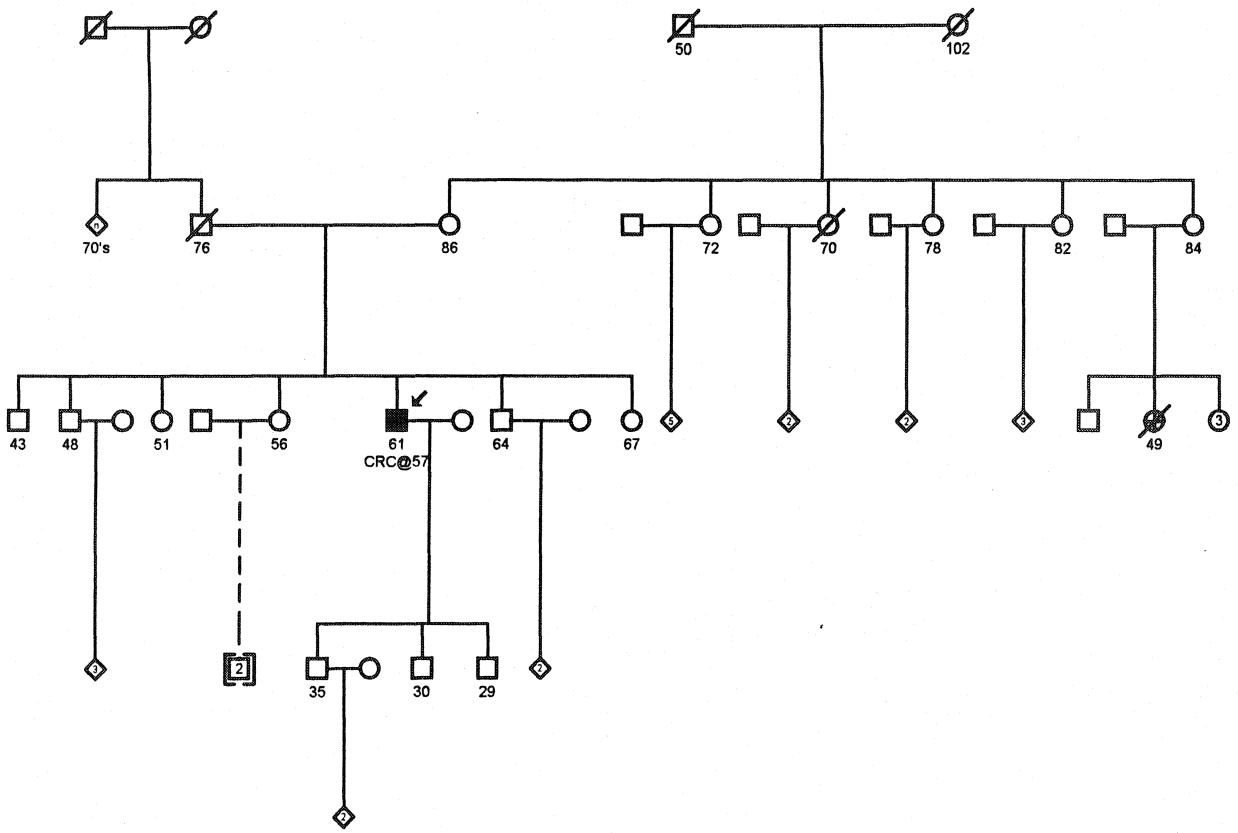
Pedigree 56 **Low-Family 121**

MSS
no loss



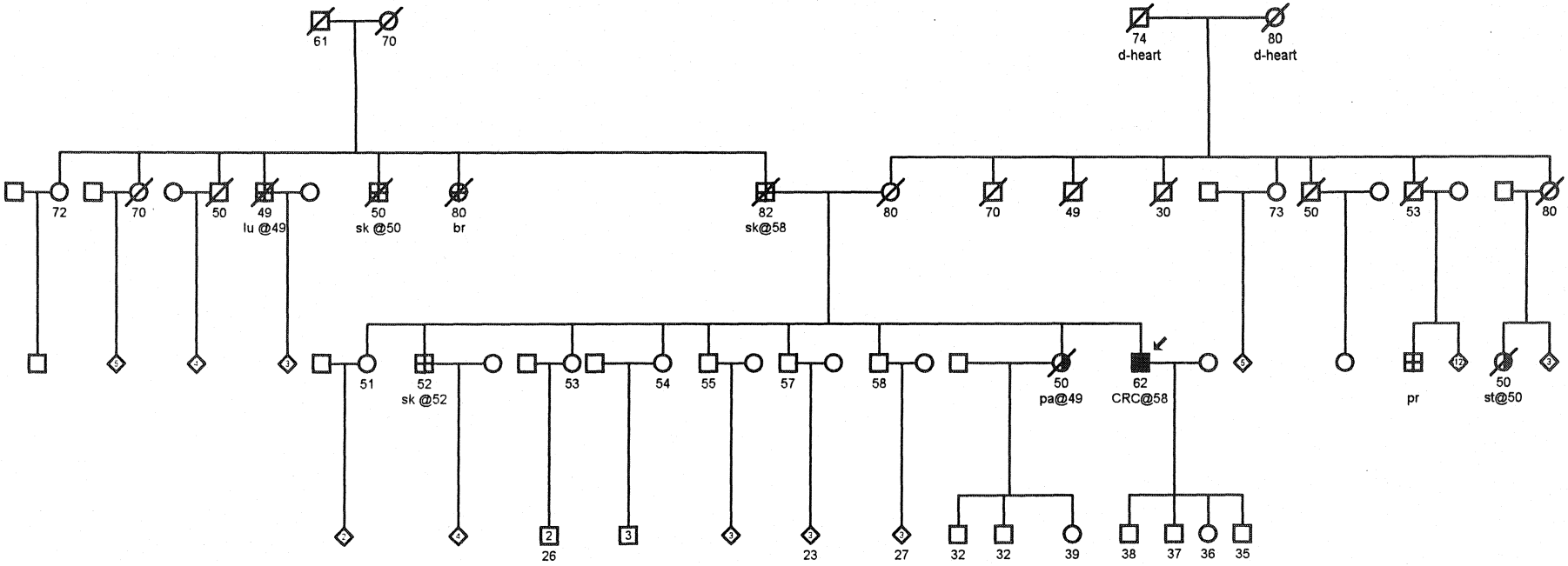
Pedigree 57 **Low-Family 125**

MSS
no loss



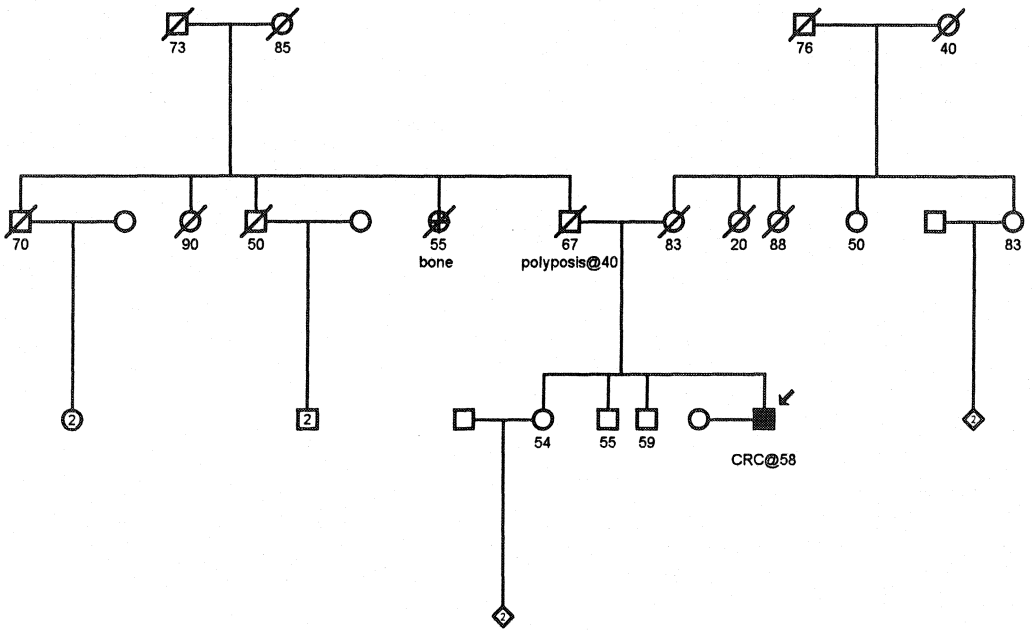
Pedigree 58 **Low-Family 161**

MSS
no loss



Pedigree 59 **Low-Family 166**

MSS
no loss



Pedigree 60 Low-Family 22

MSS
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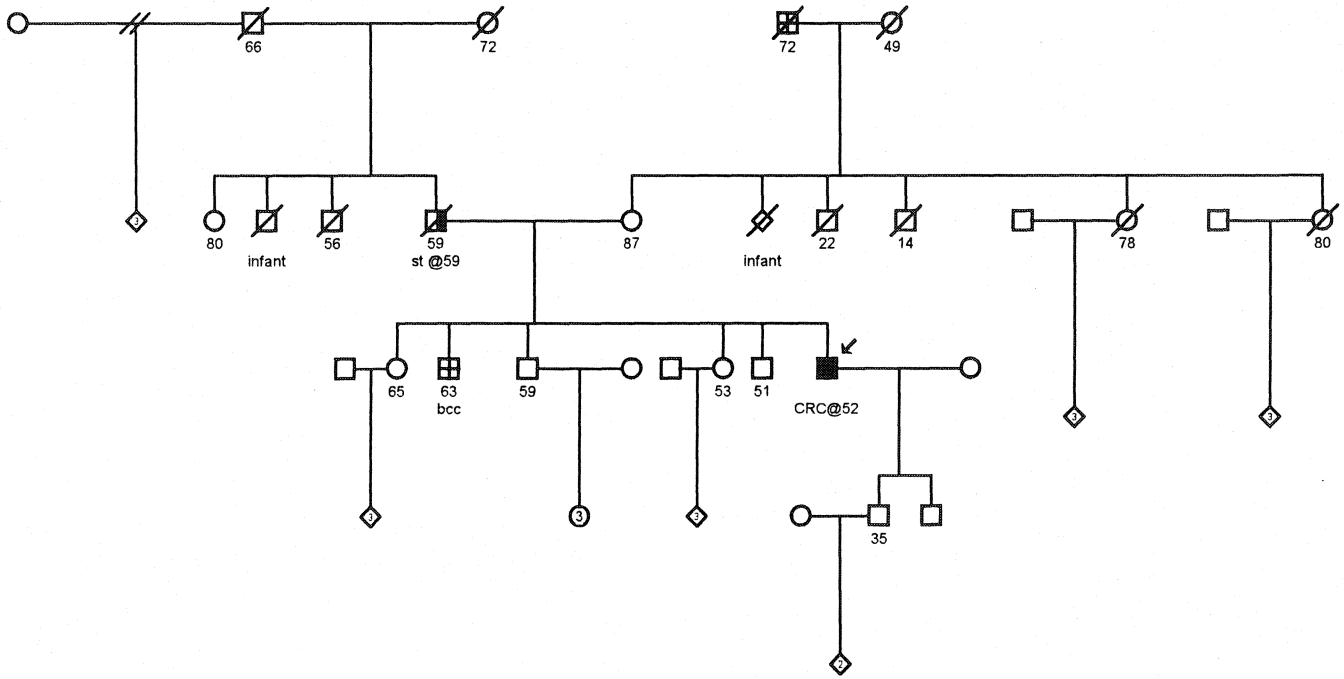
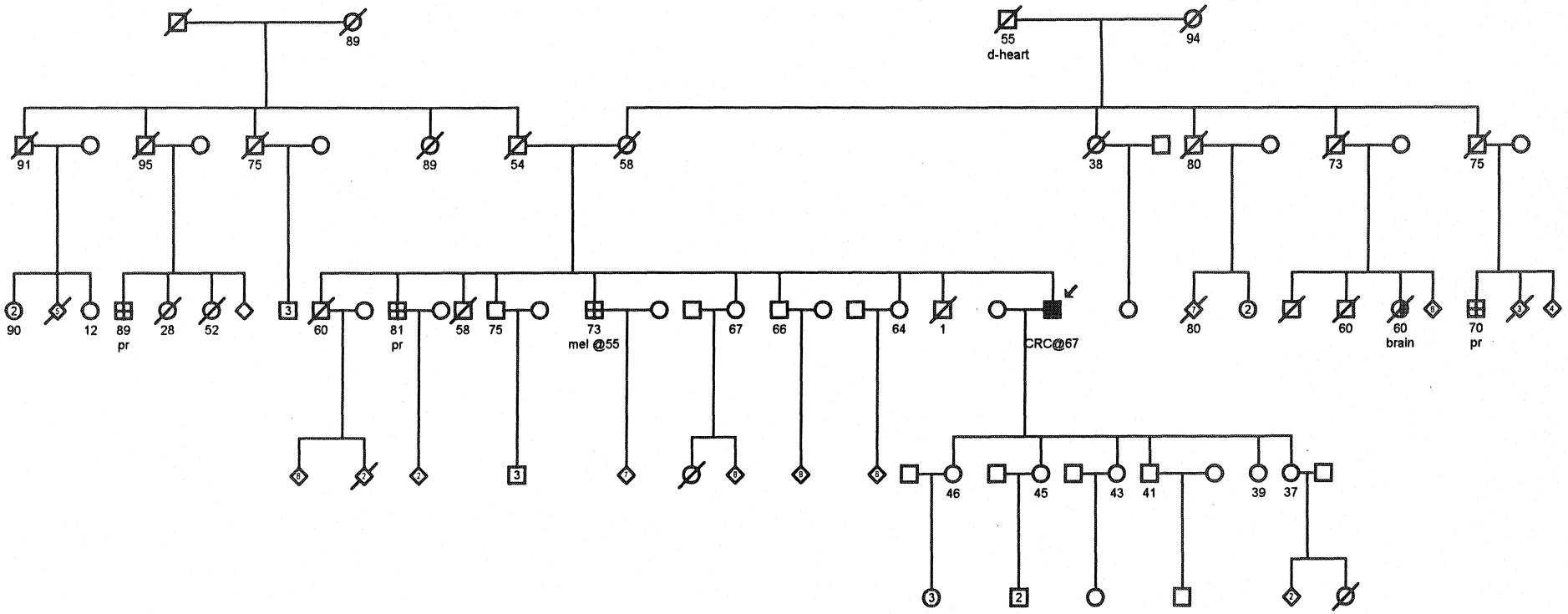


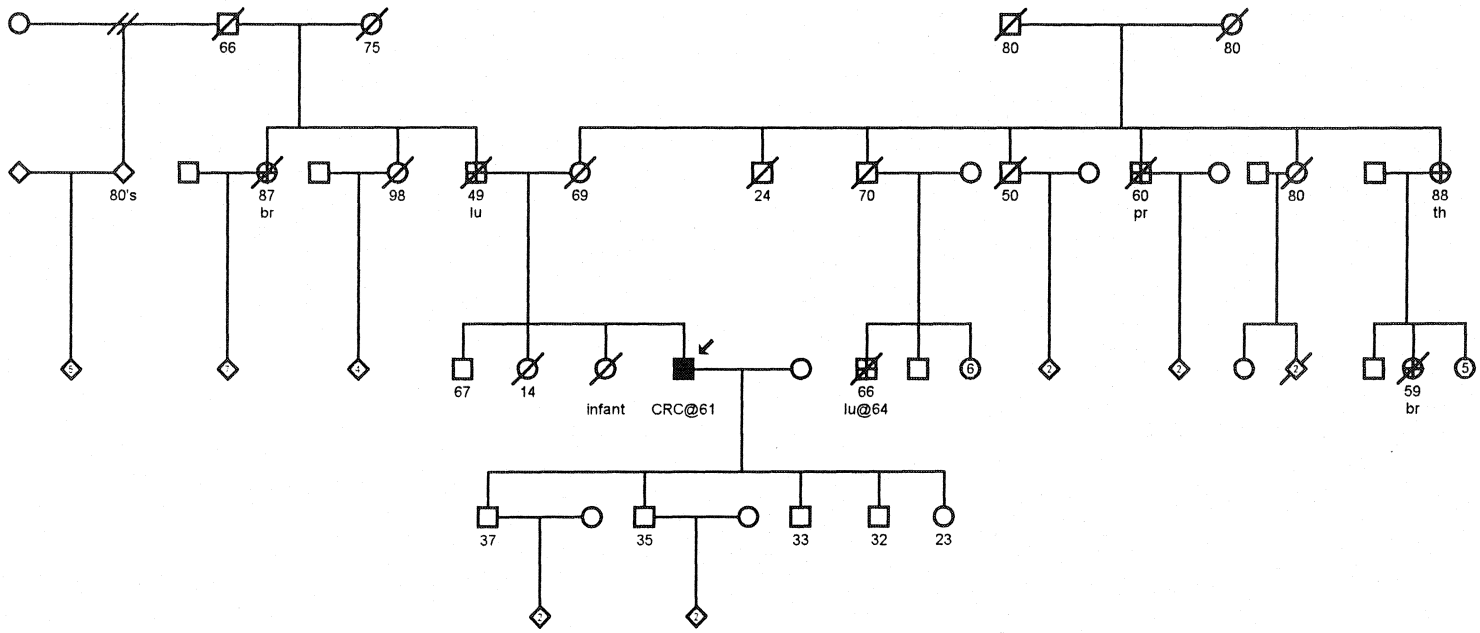
Pedigree 61 **Low -Family 24**

MSS
no loss



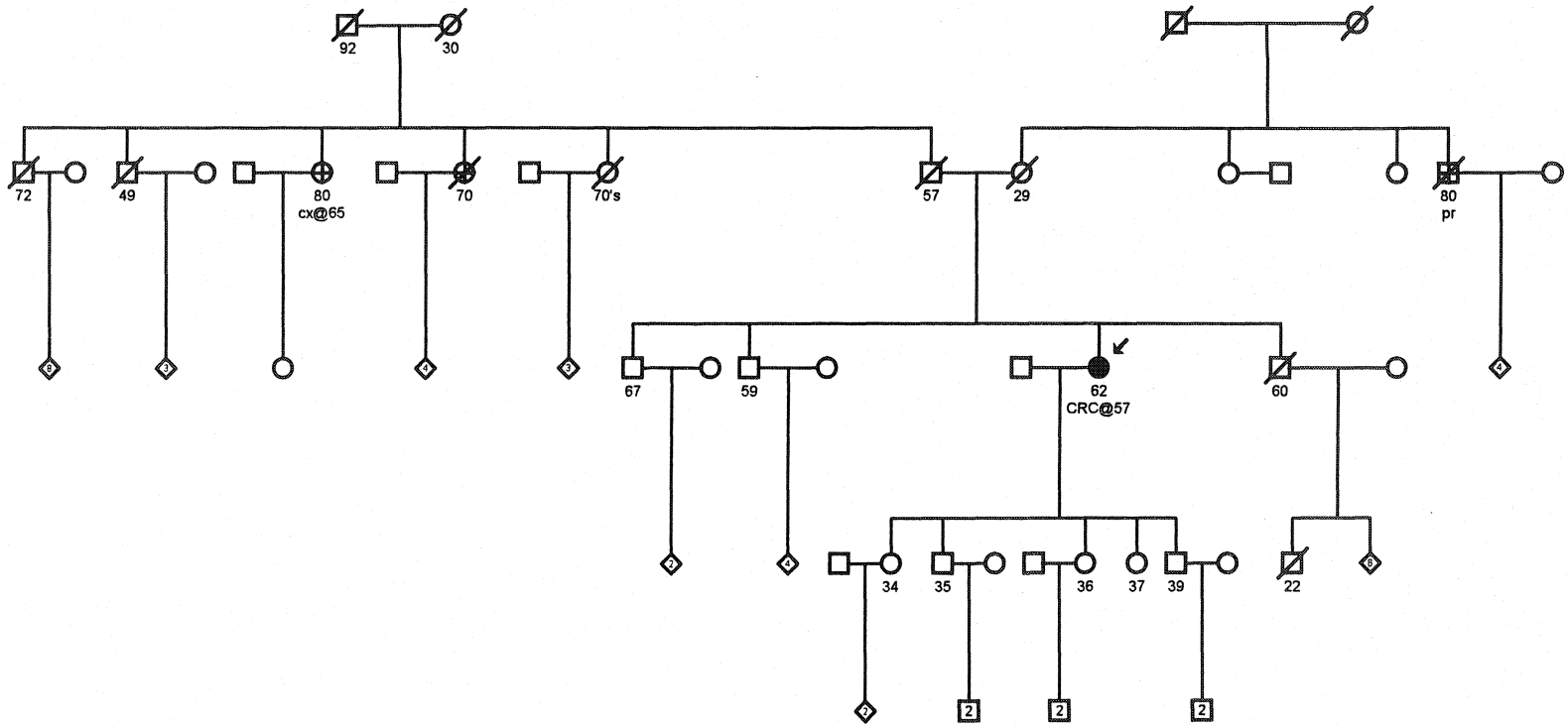
Pedigree 62 **Low-Family 41**

MSS
no loss



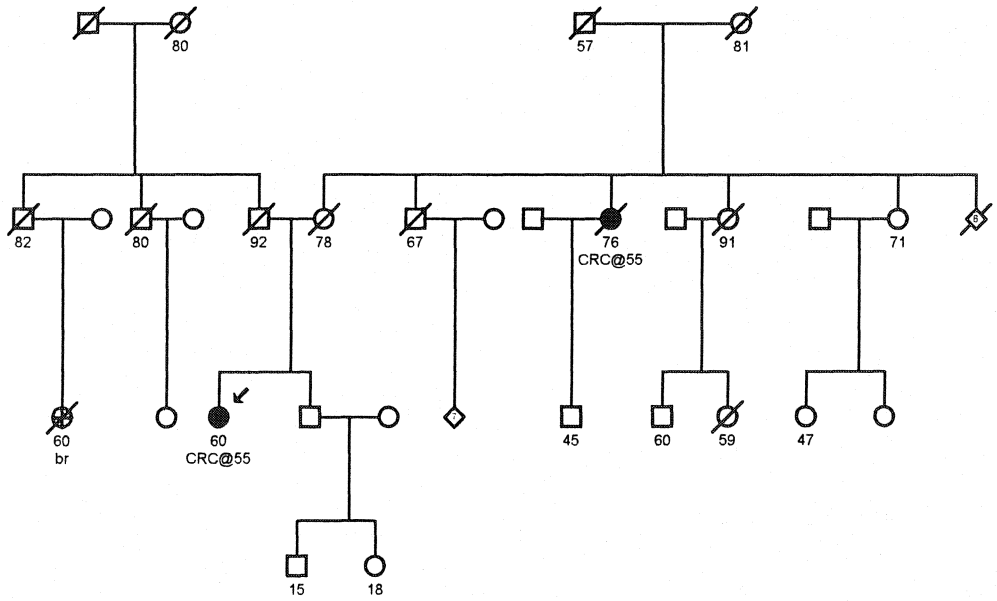
Pedigree 63 Low-Family 81

MSS
no loss



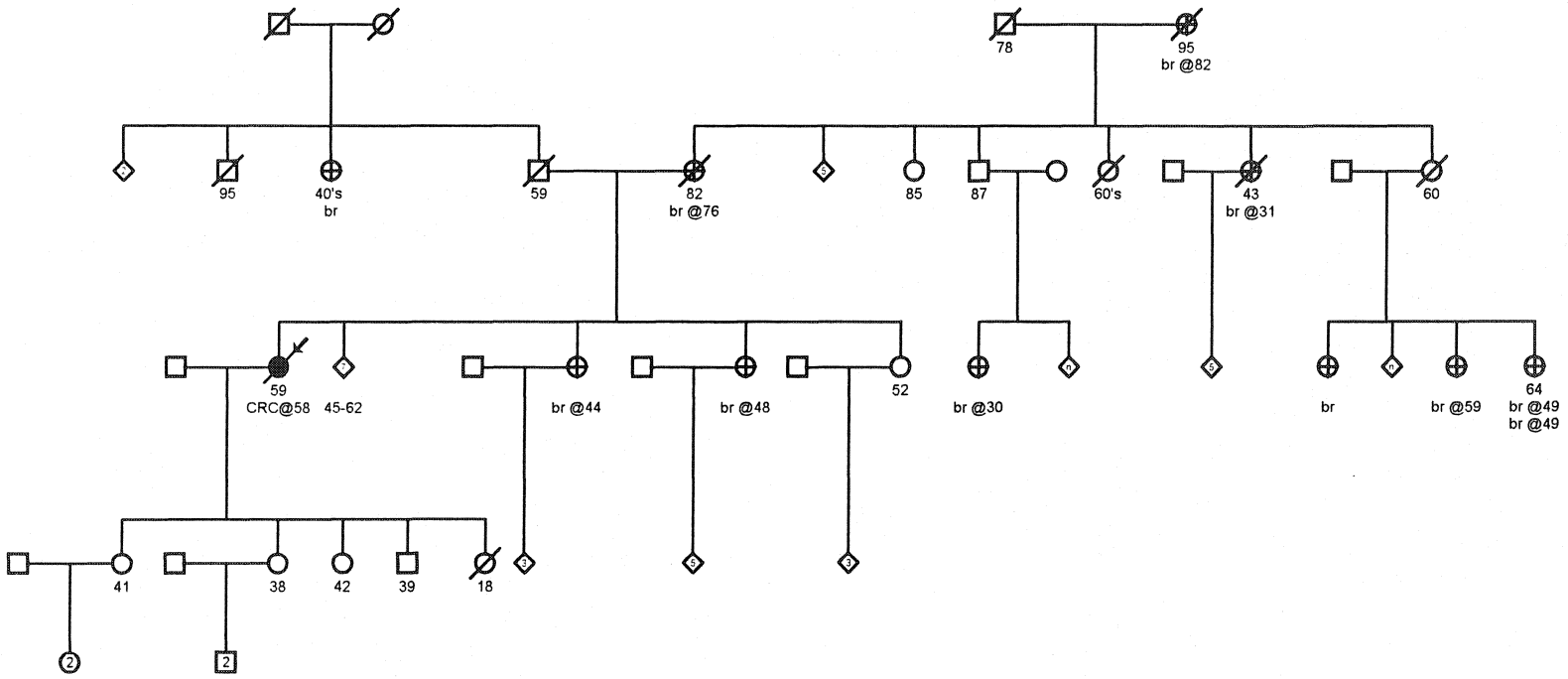
Pedigree 64 **Low-Family 92**

MSS
no loss



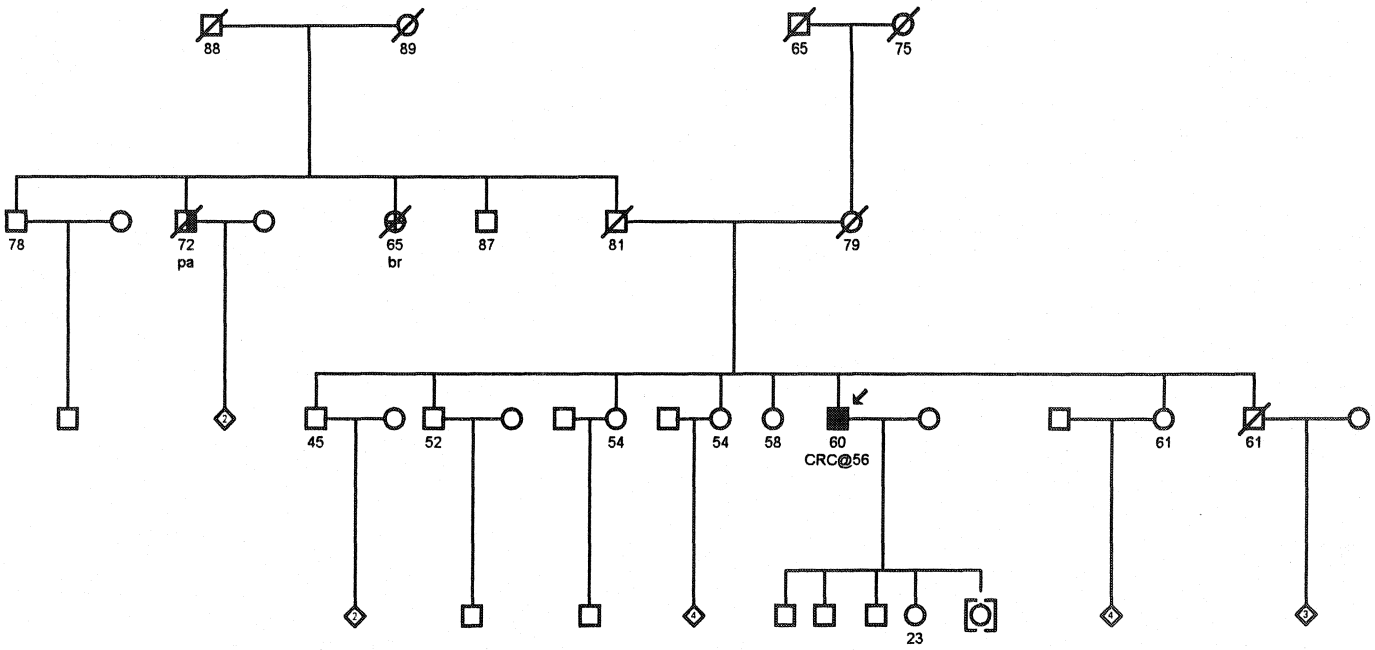
Pedigree 65 **Low-Family 115**

MSS
no loss



Pedigree 66 Low-Family 116

Pending Laboratory



Pedigree 67 Low-Family 145

Pending Laboratory

LOW RISK Uninformative families (n=12)

Proband number 65 (pedigree 68) is a 58 year old male who was diagnosed, in 1998, with a moderately differentiated cancer of the sigmoid colon at age 53. Pathology reveals the presence of two hyperplastic polyps. Previous history reports the removal of a benign mole. He was born in St. John's where he now resides. He wanted to participate in the research but at this time did not want to disclose much information therefore the pedigree drawn contains little information. The family is informative because there were insufficient family details given by the proband. The only information given was that his father died at the age of 67 following a surgery to give him a colostomy. The family is uninformative because there were insufficient family details given by the proband.

Proband number 66 (pedigree 69) is 54 year old female who was diagnosed, in 1998, with a moderately differentiated cancer of the transverse colon at age 51. She was born in St. John's where she now resides. Her mother and her father and their parents originated from Petty Harbour. The pedigree constructed consists of 57 relatives. She has 14 first degree relatives: 2 children, 10 siblings, and parents. Her mother is 74 years old. Her father died of an aneurysm at the age of 78. No cancers were reported in her first degree relatives. She has 30 second degree relatives. No cancers were reported in the family. The family is uninformative because there are less than six siblings at risk greater than 60 years old.

Proband number 68 (pedigree 70) is now deceased. He was a male who was diagnosed, in 1997, with a moderately differentiated cancer of the rectosigmoid junction

with liver metastasis at age 49. Pathology revealed the presence of 3 sessile polyps. He subsequently died a year later. His wife acted as the proxy. He was born in St. John's and resided in Mt. Pearl. Both of his parents and their parents originated from St. John's. The pedigree constructed consists of 34 relatives. He has 5 first degree relatives: 1 child, 2 siblings, and parents. His mother died of Alzheimer's at age 72. His father died from heart complications at age 72. One brother died of a stroke at age 56. No cancers were reported in his first degree relatives. He has 15 second degree relatives. A breast cancer was reported at age 65 in a maternal aunt. No other cancers were reported in the second degree relatives. There were 2 cancers reported in maternal cousins. A HNPCC cancer, CRC, at age 45, and a cancer (unknown type) at age 45 in another cousin. No other cancers were reported in the family. The family is uninformative because there were insufficient family details given by the proband.

Proband number 74 (pedigree 71) is a male who was diagnosed, in 1998, with a moderately differentiated cancer of the cecum at age 65. Chart review indicated that the proband had undergone a bowel resection in 1980. An adenoma was also reported in 1995, and a he had a polyp removed in 2001. He was born in China. His Family originated from China. His father came to St. John's in 1927, and the family followed. He did not know his grandparents. The pedigree constructed consists of 23 relatives. He has 8 first degree relatives: 3 children, 3 siblings, and parents. His father died from high blood pressure at age 66. His mother died of natural causes at age 98. No cancers were reported in his first degree relatives. He has 17 second degree relatives. There were 2 reported cancers in the family. They were a prostate cancer in a nephew, and a cancer (unknown type) in another nephew. No other cancers were reported in the family. The

family is uninformative because there were insufficient family details given by the proband.

Proband number 77 (pedigree 72) is a male who was diagnosed in 1997 with a moderately differentiated cancer of the sigmoid colon at age of 61. Chart review revealed the presence of a few polyps. He was born in Moncton, New Brunswick. He now lives in St. John's. His mother and his maternal grandfather originated from New Brunswick. His maternal grandmother originated from Prince Edward Island. His father and his paternal grandfather originated from New Brunswick. His maternal grandmother originated from Nova Scotia. The pedigree constructed consists of 23 relatives. He has 8 first degree relatives: 5 children, 1 sibling, and parents. His mother died of Alzheimer's at age 86. His father died of heart complications at 84. No cancers were reported in his first degree relatives. He has 13 second degree relatives. A breast cancer was reported in a paternal aunt at age 59. No other cancers were reported in his second degree relatives. A breast cancer was reported in a maternal cousin at age 61. No other cancers were reported in the family. The family is uninformative because there are less than six siblings at risk.

Proband number 123 (pedigree 73) is a 39 year old female who was diagnosed, in 1998, with a moderately differentiated cancer of the sigmoid colon at age 36. She was born in Carbonear and now resides in Victoria. Her mother and her maternal grandparents originated from Whitbourne. Her father originated from Bell Island. Her paternal grandmother originated from Nova Scotia. The origins of her paternal grandfather originated are unknown. The pedigree constructed consists of 46 relatives. She has 13 first degree relatives: 2 children, 9 siblings, and parents. Her mother died

from a rare disease (unknown) at age 52. Her father is still alive and is 73 years old. No cancers were reported cancers in her first degree relatives. She has 32 second degree relatives. Leukemia was reported in a maternal uncle who died at age 70. No other cancers were reported in her second degree relatives. Leukemia was reported in a maternal cousin, who died at age 25. No other cancers were reported in the family. The family is uninformative because there are less than six siblings at risk greater than 60 years old.

Proband number 128(pedigree 74) is a 50 year old male who was diagnosed, in 1998, with a moderately differentiated cancer of the sigmoid at age 46. He was born in Rumania. The pedigree constructed consists of 11 relatives. He has first degree relatives: 2 children, 1 sibling, and parents. His father was diagnosed with a prostate cancer at age 74. No other cancers were reported cancers in first degree relatives. He has 6 second degree relatives. A HNPCC cancer, CRC, was reported in his paternal grandmother at age 80. A prostate cancer was reported in his paternal grandfather at age 85. No other cancers were reported in the family. This proband was previously referred to the Medical Genetics Clinic. The family is uninformative because there are less than six siblings at risk.

Proband number 133 (pedigree 75) is a 68 year old male who was diagnosed with a cancer of the sigmoid at age 62. He was born in St. John's. His Parents and grandparents both originated from St. John's. The pedigree constructed consists of 37 relatives. He has 10 first degree relatives, 4 children, 4 siblings (half), and parents. His mother died in her late 30's from a "tedious disease" when the proband was just 4. He did not know his mother's family. His father died at from heart complications at age 68.

No cancers were reported in his first degree relatives. He has 22 second degree relatives. HNPCC cancers, CRC are reported in a paternal aunt who died in her 70's and in a paternal cousin at age 70. No other cancers were reported are reported in the family. The family is uninformative because there were insufficient family details given by the proband.

Proband number 149 (pedigree 76) is a male who is now deceased. He was diagnosed in 1998 with an undifferentiated cancer of the rectosigmoid junction with liver metastasis at age 47. He died shortly after he was diagnosed. His wife was identified as his proxy. Follow-up with the proband's siblings was attempted but not successful. He was born in Kilbride and resided in the Goulds. His mother originated from Kilbride and his father originated from Upper Gullies. His grandparent's demographics were not given. The pedigree constructed consists of 38 relatives. He has 11 first degree relatives: 2 children, 7 siblings, and parents. His mother died of heart complications at age 71 and his father died of a stroke at age 81. A sister died from a cancer (primary unknown, but CRC was implied) that had metastasised to her liver and pancreas at age 56. No other cancers were reported in his first degree relatives. He has 27 second degree relatives. No other cancers were reported in the family. The family is uninformative because there were insufficient family details given by the proband.

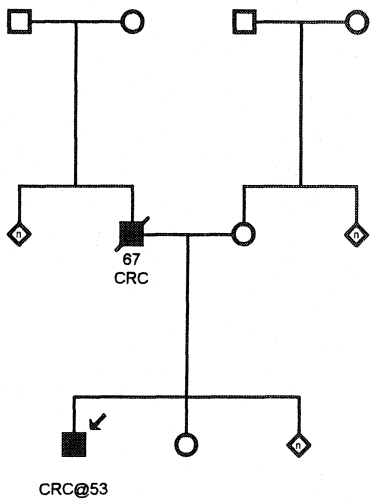
Proband number 164 (pedigree 77) is a male who is now deceased. He was diagnosed, in 1997, with cancer of the rectosigmoid at age 47. He was diagnosed with liver and lung metastasis 2 years later and died in 2000. His wife was identified as his proxy. He was born and resided in Placentia. His mother and maternal grandfather originated from Presque, Placentia Bay. His maternal grandmother originated from

Paradic, Placentia Bay. His father and paternal grandparents originated from Placentia. The pedigree constructed consists of 44 relatives. He has 4 first degree relatives, 2 children, and parents. His mother died from HNPCC cancer, CRC at age 62 and his father died from heart complications at age 83. No other cancers were reported in his first degree relatives. He has 13 second degree relatives. No cancers were reported in second degree relatives. There were reported cancers (type unknown) in 3 paternal cousins. No other cancers were reported in the family. The family is uninformative because there were insufficient family details given by the proband.

Proband number 177 (pedigree 78) is a female who is now deceased. She was diagnosed, in 1997, with a moderately differentiated cancer of the colon with ovary, liver, and uterine metastasis at age 50. Her daughter was identified as her proxy. She was born in St. John's and resided in Mt. Pearl. Her parents and her grandparents originated from St. John's. The pedigree constructed consists of 23 relatives. She has 8 first degree relatives: 2 children, 4 siblings, and parents. Her mother died of breast cancer metastasis at age 76. Her father died of heart complications at age 70. A sister was diagnosed with a kidney cancer at age 66. No other cancers were reported in first degree relatives. She has 15 second degree relatives. No other cancers were reported in the family. The family is uninformative because there are less than six siblings at risk greater than 60 years old.

Proband number 28(pedigree 79) is a 56 year old male who was diagnosed, in 1998, with a moderately differentiated cancer of the descending colon at age 52. The proband was born in Twillingate and he now resides in St. John's. His mother and maternal grandfather originated from Derry, Ireland. His maternal grandmother originated from Scotland. The pedigree drawn consists of 33 relatives. He has 6 first

degree relatives: 2 child, 2 siblings, and parents. His mother is alive and is 71 years old. His father died at 73 due to heart disease. No cancers were reported in his first degree relatives. He had 18 second degree relatives. Breast cancer was reported in a maternal aunt at less than 30 years old. No other cancers were reported in the family. The family is uninformative because there were insufficient family details given by the proband.



Pedigree 68 Low uninform-Family 65

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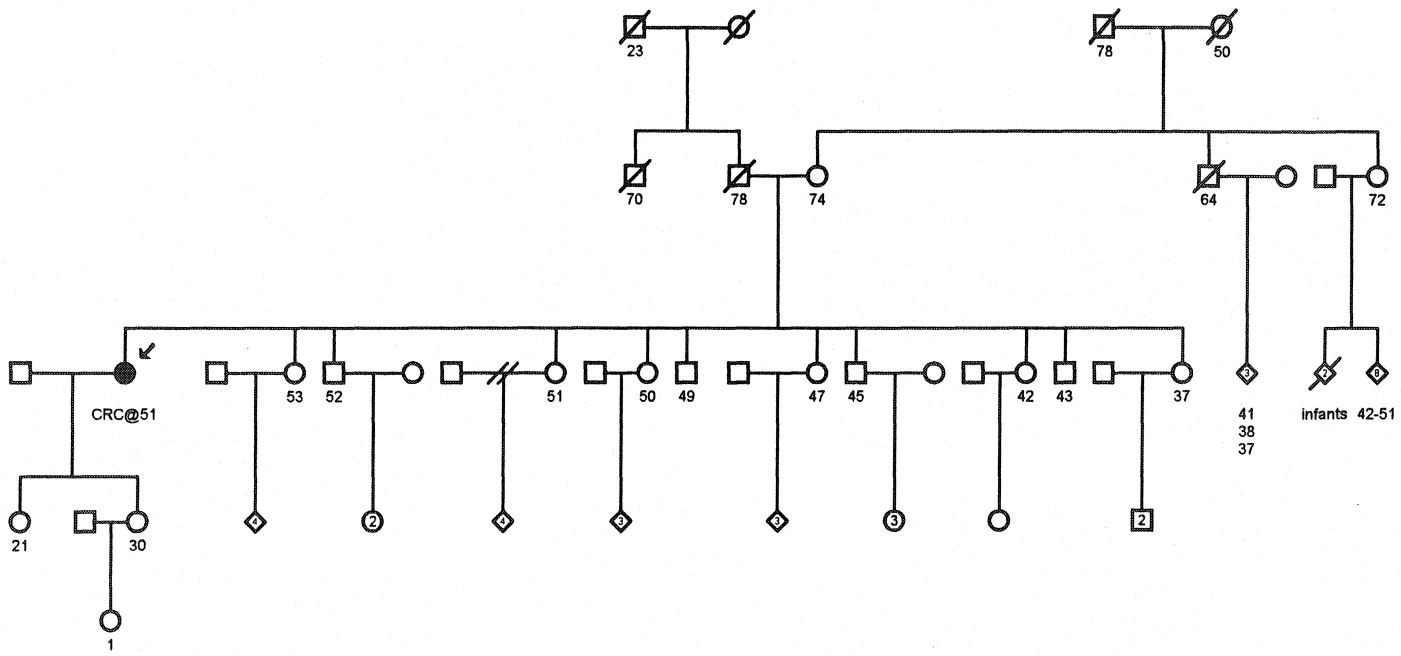
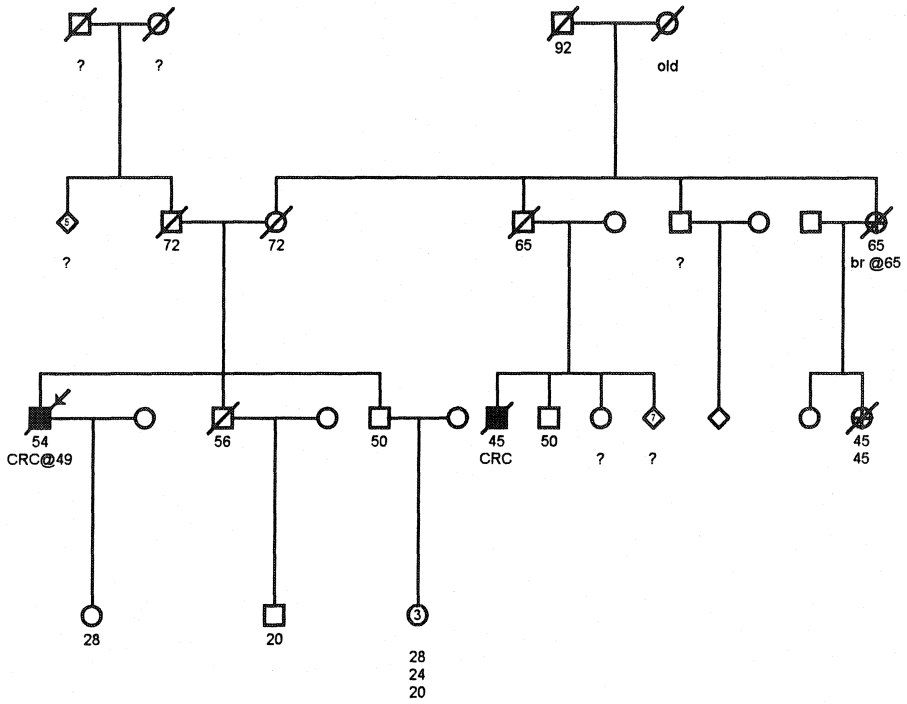


Pedigree 69 **Low uninform-Family 66**

MSS
no loss



Pedigree 70 Low uniform-Family 68

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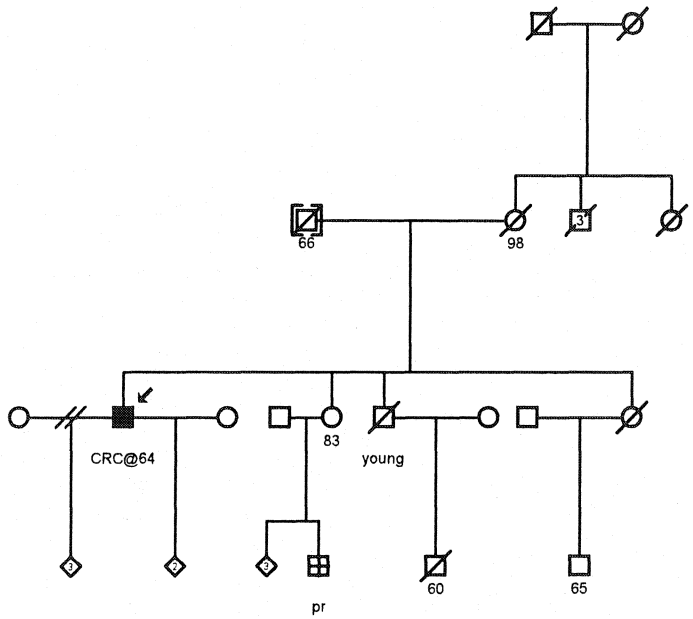
no loss



Pedigree 71 Low uninform-Family 74

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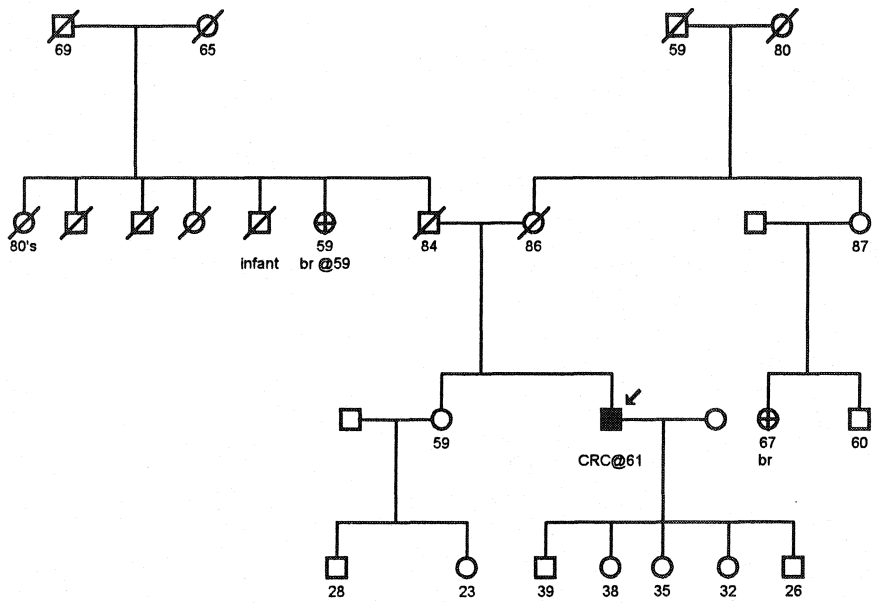

Pedigree 72 Low uninform-Family 77

MSS
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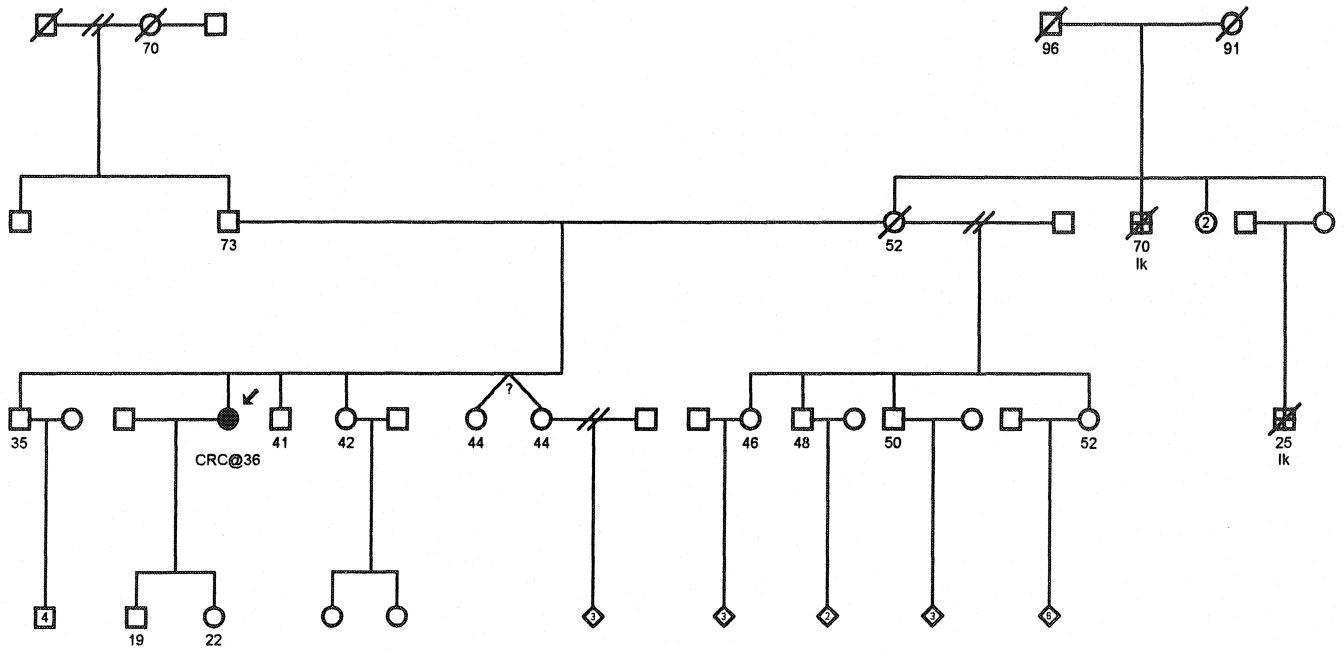

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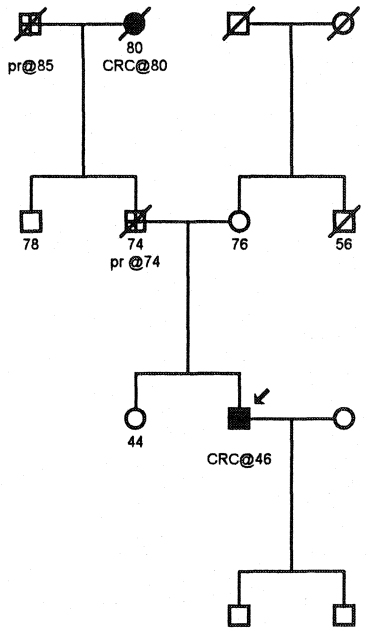

Pedigree 74 Low uninform-Family 128

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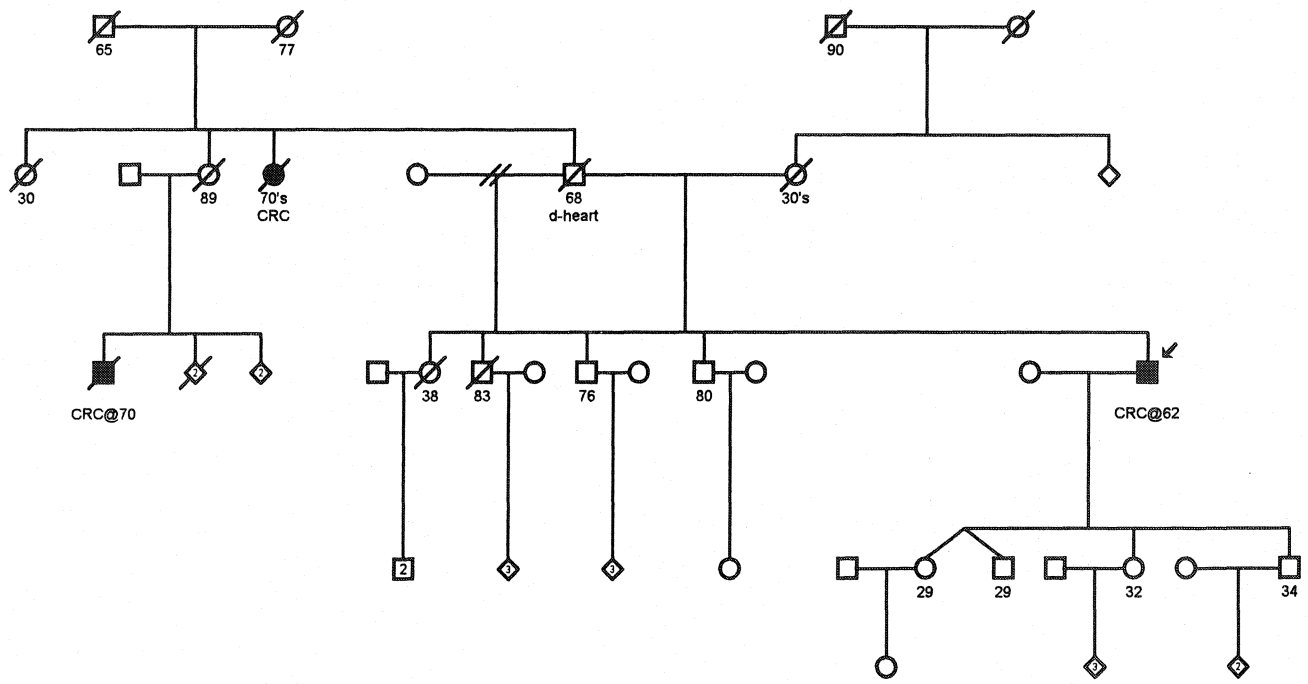

Pedigree 75 Low uninform-Family 133

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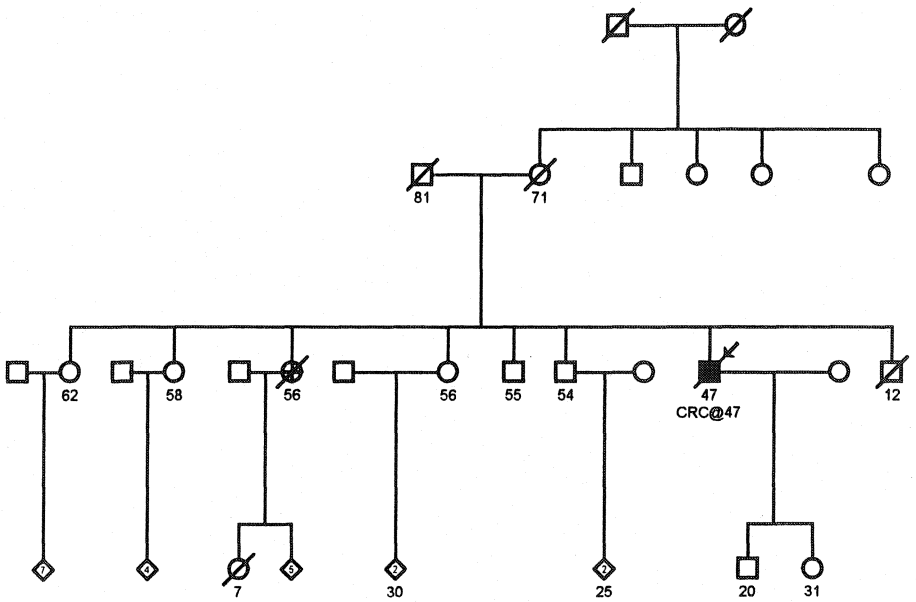

Pedigree 76 **Low uninform-Family 149**

MSS
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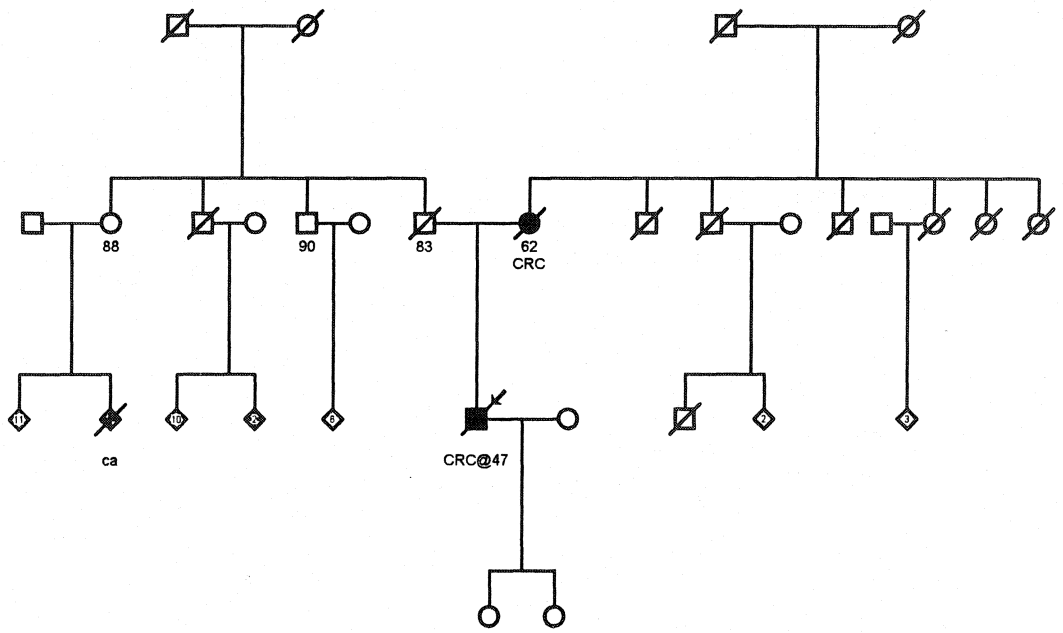

Pedigree 77 Low uninform-Family 164

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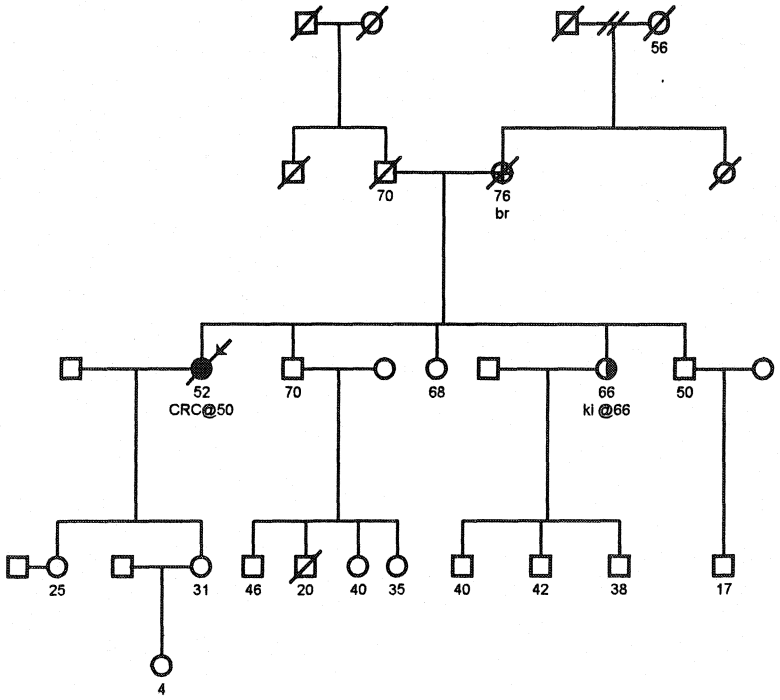
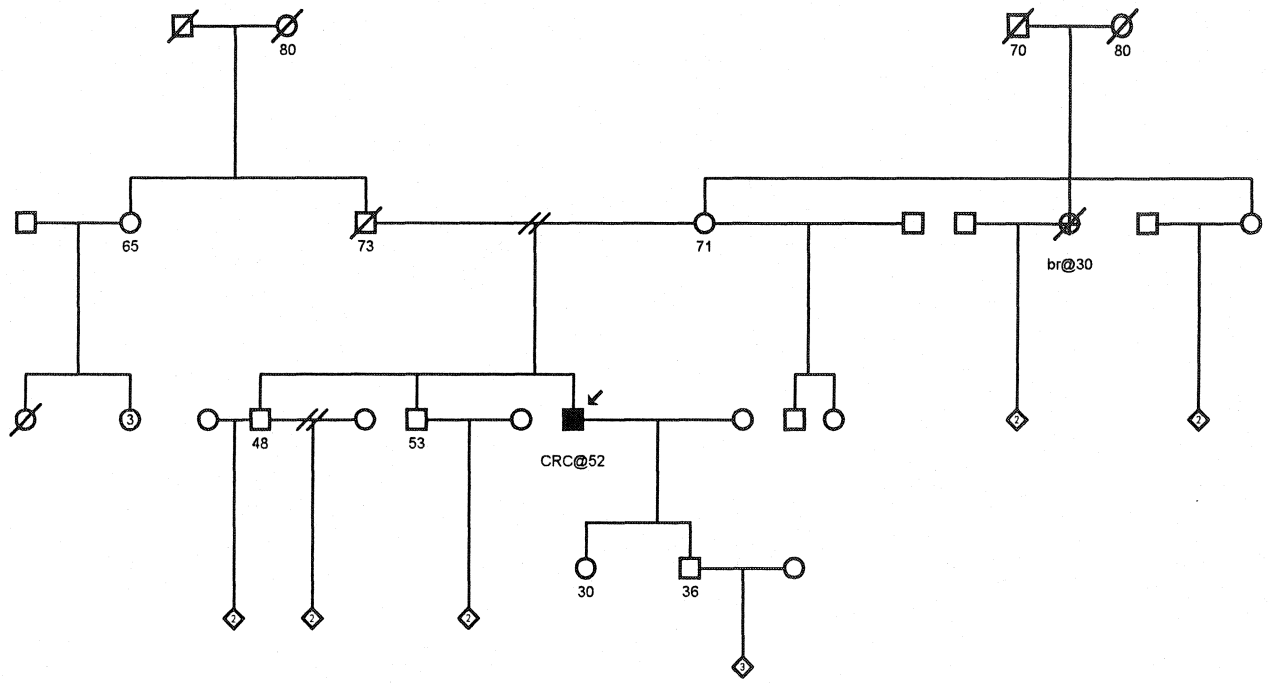


Pedigree 78 Low uninform-Family 177

MSS
no loss



Pedigree 79 **Low uninform-Family 28**

MSS
no loss

APPENDIX B: Human Investigation Committee Approval

APPENDIX C: Study letter to physician

March 15th, 2001

«Title» «FirstName» «LastName»
«Address1»
«Address2»
«City»,«State»
«PostalCode»

Dear Dr. Becker:

RE: THE GENETICS OF COLO-RECTAL CANCER IN NEWFOUNDLAND

I am writing on behalf of a research team of Memorial University to request your participation in this study of the genetics of colo-rectal cancer in Newfoundland. The investigators include Dr. P. Parfrey, Dr. J. Green, Dr. R. Green, Dr. B. Younghusband and myself. Much of the work will be done by graduate students – Fiona Curtis and Angela Hyde, who will have direct contact with patients and their families.

This portion of the study wishes to determine the contribution of genetic factors to the incidence of colo-rectal cancer in Newfoundland. It will involve direct contact with patients or their families by the graduate students who will take detailed family histories. Patients and family members may be asked to contribute blood samples. This has been approved by the Human Investigation Committees of Memorial University of Newfoundland and the Health Care Corporation of St. John's. Part of the protocol requires that the initial contact with the patient should be initiated by their attending physician. I am writing to enquire whether you would be willing to sign a standardized letter (sample enclosed) which will be sent to your patients or their families asking for their participation in this research.

If you agree to participate, addressed postage paid letters will be provided for you to sign which will then be sent to patients or their families. Patients will be asked to respond either directly to the research team or your office.

.../2

Dr.
March 15th, 2001
RE: The Genetics of Colo-rectal Cancer in Newfoundland

We believe that this research may have important benefits in elucidating the epidemiology of colo-rectal cancer in Newfoundland and may result in significant clinical benefits for patients and their families.

Thank you for considering this request. Please let me know if you are willing to participate at your earliest convenience. If you have any questions or concerns please contact me directly.

Yours sincerely,

W.G. Pollett, MD, FRCSC
Professor & Chair
Discipline of Surgery
Memorial University of NF
Health Sciences Centre

PHONE: (709) 737-6612
FAX: (709) 737-5050

e-mail address:
wpollett@morgan.ucs.mun.ca

WGP/sw

Enclosure

APPENDIX D: Physician letter to proband and to proxy

Genetics of Colorectal Cancer in Newfoundland Research Project

DATE:_____

DEAR

Researchers at Memorial University are interested in learning more about the hereditary nature of colon and rectal cancer. Most cancers happen by chance but some kinds of cancer are inherited. Most colon and rectum cancers are diagnosed in people over 65. About 10% of colon and rectum cancers are inherited, and they often occur before the age of 50.

They need to find out how many people with a diagnosis of colon and rectum cancer in a single year have the inherited kind. If a family is found to have an inherited kind then research can be used to identify the kind of gene that may cause the cancer in that family. This could lead to an earlier diagnosis and treatment for families that have the gene.

I would like to ask you to take part in the study on behalf of the research team (led by Dr Patrick Parfrey, Dr. William Pollett, and Dr. Jane Green). I know that your wife, Dorothy was diagnosed with colon and rectum cancer. Family members can give us valuable information for this research. Your participation is entirely voluntary. You may decide not to take part or you may withdraw from the study at any time.

If you wish to take part in the study you may call the research team, collect at (709) 777-7622, or you can contact our office and we will pass your name on to the research team and they will contact you by telephone. They will ask you for a convenient time to meet with you. During this meeting the researcher will ask you to help trace your family history. If there seems to be a family history of colon and rectum cancer, you and your family members will be asked to have a simple blood test. This test may or may not be able to tell if you have a gene that put you at an increased risk for colon and rectum cancer.

If you do not wish to take part in the research project please call my office between the hours of _____, and give your name to my secretary.

Your participation would be greatly appreciated. With your help the research team hopes to learn more about colon and rectum cancer, the role genes play, and how often colon and rectum cancer occurs in Newfoundland.

Yours truly,

_____

HealthCare
Corporation of St. John's

Genetics of Colorectal Cancer in Newfoundland Research Project

DATE:_____

DEAR_____

Researchers at Memorial University are interested in learning more about the hereditary nature of colon and rectal cancer. Most cancers happen by chance but some kinds of cancer are inherited. Most colon and rectum cancers are diagnosed in people over 65. About 10% of colon and rectum cancers are inherited, and they often occur before the age of 50.

They need to find out how many people with a diagnosis of colon and rectum cancer in a single year have the inherited kind. If a family is found to have an inherited kind then research can be used to identify the kind of gene that may cause the cancer in that family. This could lead to an earlier diagnosis and treatment for families that have the gene.

I would like to ask you to take part in the study on behalf of the research team (led by Dr Patrick Parfrey, Dr. William Pollett, and Dr. Jane Green). I know that your _____, _____ was diagnosed with colon and rectum cancer. Family members can give us valuable information for this research. Your participation is entirely voluntary. You may decide not to take part or you may withdraw from the study at any time.

If you wish to take part in the study you may call the research team, collect at (709) 777-7622, or you can contact our office and we will pass your name on to the research team and they will contact you by telephone. They will ask you for a convenient time to meet with you. During this meeting the researcher will ask you to help trace your family history. If there seems to be a family history of colon and rectum cancer, you and your family members will be asked to have a simple blood test. This test may or may not be able to tell if you have a gene that put you at an increased risk for colon and rectum cancer.

If you do not wish to take part in the research project please call my office between the hours of _____, and give your name to my secretary.

Your participation would be greatly appreciated. With your help the research team hopes to learn more about colon and rectum cancer, the role genes play, and how often colon and rectum cancer occurs in Newfoundland.

Yours truly,

_____

APPENDIX E: Consent forms

Study consent form
Newfoundland and Labrador Medical Genetics Clinic consent form
Release of medical information form

FACULTY OF MEDICINE - MEMORIAL UNIVERSITY OF NEWFOUNDLAND
AND
HEALTH CARE CORPORATION OF ST. JOHN'S

Consent To Participate In Bio-medical Research

TITLE: The genetics of colorectal cancer in Newfoundland

INVESTIGATOR(S): Fiona Curtis, Angela Hyde, Dr. P. Parfrey, Dr. J. Green, Dr. B. Pollett, Dr. R. Green, Dr. B. Younghusband

You have been asked to take part in a research study. Participation in this study is entirely voluntary. You may decide not to take part or may withdraw from the study at any time without affecting your normal treatment.

Information obtained from you or about you during this study, which could identify you, will be kept confidential by the investigator(s). The investigator will be available during the study at all times should you have any problems or questions about the study.

1. Purpose of study:

   Most cancers affect individuals, but some cancers occur in several family members. These cancers may be inherited, that is they "run in the family" as a result of changes in some genes. The purpose of this study is to find out how many people with a diagnosis of colorectal cancer have an inherited form of the disease and to develop laboratory tests to identify these cases.

2. Description of procedures and tests:

   You will meet with the investigator at your convenience in person or by telephone if necessary. You will help trace your family medical history. If there is an indication of inherited colorectal cancer in your family then you will receive a referral to the Medical Genetics program for yourself and your family members for further consultation. We will ask your permission to review your medical records, obtain some of your cancer tissue which was removed during surgery and if you agree, a blood sample will be taken for further genetic studies.

3. Duration of participant's involvement:

   The overall duration of the project is 2 years. Your direct involvement will be to meet with the researcher, in person or by telephone, to discuss your family history and to give a blood sample.

4. Possible risks, discomforts, or inconveniences:

- The interview may be an inconvenience.
- A blood sample taken may lead to some bruising.

5. Benefits which the participant may receive:

If there is evidence of a hereditary cancer syndrome in your family, you will be given a referral to a geneticist which could then lead to screening and early detection and treatment of cancer in your family members.

6. Alternative procedures or treatment for those not entering the study:

If you decide not to enter this study, you will receive your normal care from your physician.

7. Liability statement.

Your signature indicates your consent and that you have understood the information regarding the research study. In no way does this waive your legal rights nor release the investigators or involved agencies from their legal and professional responsibilities

8. Statement on genetic studies:

In order to interpret the results of the genetic research, we need to have correct information about parents. Sometimes the research shows new information about birth parents. This could happen in the case of an adoption or a mistake in the identity of a mother or father. This information will not be given to anyone, including yourself or family members.

9. Future use of tissue/DNA samples

In order to preserve a valuable resource, your tissue/DNA samples may be stored at the end of this research project. It is possible that these samples may be useful in a future research project which may or may not be related to the current research project. Any future research would have to be approved by a Research Ethics Board (REB).

Please tick **one** of the following options:

1. ☐ I agree that my tissue/DNA samples with my name can be used for any approved research project without asking me again.

2. ☐ I agree that my tissue/DNA samples with my name can be used for any approved research project but only if I am contacted again to give consent for the new project.

3. ☐ I agree that my tissue/DNA samples with my name can be used for any approved research project but only if my name* cannot be linked to the sample.

4. ☐ Under no circumstances may my tissue/DNA samples be used for future research. My samples must be destroyed at the end of the present project.

*Includes name, MCP number or any other identifying information.

The tissue/DNA samples from this study will be stored in St. John's, NF (city/province) for (approximately __ years/for an indefinite period of time).

Signature: _____ Date: _____

Witness: _____

Signature Page

Title of Project: The Genetics of Colorectal cancer in Newfoundland

Name of Principal Investigator: Fiona Curtis, DR. Jane Green (supervisor)

To be signed by participant

I, , the undersigned, agree to my participation or to the participation of (my child, ward, relative) in the research study described above.

Any questions have been answered and I understand what is involved in the study. I realise that participation is voluntary and that there is no guarantee that I will benefit from my involvement.

I acknowledge that a copy of this form has been given to me.

(Signature of Participant) (Date)

(Signature of Witness) (Date)

To be signed by investigator

To the best of my ability I have fully explained the nature of this research study. I have invited questions and provided answers. I believe that the participant fully understands the implications and voluntary nature of the study.

(Signature of Investigator) (Date)

Phone Number

Assent of minor participant (if appropriate)

(Signature of Minor Participant) (Age ____)

Relationship to Participant Named Above

FACULTY OF MEDICINE - MEMORIAL UNIVERSITY OF NEWFOUNDLAND
AND
HEALTH CARE CORPORATION OF ST. JOHN'S

Consent To Participate In Bio-medical Research

TITLE: The genetics of colorectal cancer in Newfoundland

INVESTIGATOR(S): Fiona Curtis, Angela Hyde, Dr. P. Parfrey, Dr. J. Green, Dr. B. Pollett, Dr. R. Green, Dr. B. Younghusband

You have been asked to take part in a research study on behalf of your family member, _____ _____ . Participation in this study is entirely voluntary. You may decide not to take part or may withdraw from the study at any time.

Information obtained from you or about you and family members during this study, which could identify you and other family members, will be kept confidential by the investigator(s). The investigator will be available during the study at all times should you have any problems or questions about the study.

1. Purpose of study:

   Most cancers affect individuals, but some cancers occur in several family members. These cancers may be inherited, that is they "run in the family" as a result of changes in some genes. The purpose of this study is to find out how many people with a diagnosis of colorectal cancer have an inherited form of the disease and to develop laboratory tests to identify these cases.

2. Description of procedures and tests:

   You will meet with the investigator at your convenience in person or by telephone if necessary. You will help trace your family medical history. If there is an indication of inherited colorectal cancer in your family then you will receive a referral to the Medical Genetics program for yourself and your family members for further consultation. We will ask your permission to review your _____ medical records and permission to obtain some of your _____ cancer tissue which was removed during surgery.

3. Duration of participant's involvement:

   The overall duration of the project is 2 years. Your direct involvement will be to meet with the researcher, in person or by telephone to discuss your family history. If you are willing a blood sample may also be taken.

4. Possible risks, discomforts, or inconveniences:

- The interview may be an inconvenience.
- A blood sample taken may lead to some bruising.

5. Benefits which the participant may receive:

If there is evidence of a hereditary cancer syndrome in your family, you will be given a referral to a geneticist which could then lead to screening and early detection and treatment of cancer in you and your family members.

6. Alternative procedures or treatment for those not entering the study:

NA.

7. Liability statement.

Your signature indicates your consent and that you have understood the information regarding the research study. In no way does this waive your legal rights nor release the investigators or involved agencies from their legal and professional responsibilities

8. Statement on genetic studies:

In order to interpret the results of the genetic research, we need to have correct information about parents. Sometimes the research shows new information about birth parents. This could happen in the case of an adoption or a mistake in the identity of a mother or father. This information will not be given to anyone, including yourself or family members.

9. Future use of tissue/DNA samples

In order to preserve a valuable resource, your _____ tissue/DNA samples may be stored at the end of this research project. It is possible that these samples may be useful in a future research project which may or may not be related to the current research project. Any future research would have to be approved by a Research Ethics Board (REB).

Please tick **one** of the following options:

1. ☐ I agree that my _____ tissue/DNA samples with my _____ name can be used for any approved research project without asking me again.

2. ☐ I agree that my _____ tissue/DNA samples with my _____ name can be used for any approved research project but only if I am contacted again to give consent for the new project.

3. ☐ I agree that my _____ tissue/DNA samples with my _____ name can be used for any approved research project but only if my _____ name* cannot be linked to the sample.

4. ☐ Under no circumstances may my _____ tissue/DNA samples be used for future research. My _____ samples must be destroyed at the end of the present project.

*Includes name, MCP number or any other identifying information.

The tissue/DNA samples from this study will be stored in St. John's, NF (city/province) for (approximately __ years/(for an indefinite period of time)).

Signature: _____ Date: _____

Witness: _____

Signature Page

Title of Project: The Genetics of Colorectal Cancer in Newfoundland

Name of Principal Investigator: Fiona Curtis, DR. Jane Green (supervisor)

To be signed by participant

I, , the undersigned, agree to my participation or to the participation of (my child, ward, relative) in the research study described above.

Any questions have been answered and I understand what is involved in the study. I realise that participation is voluntary and that there is no guarantee that I will benefit from my involvement.

I acknowledge that a copy of this form has been given to me.

(Signature of Participant) (Date)

(Signature of Witness) (Date)

To be signed by investigator

To the best of my ability I have fully explained the nature of this research study. I have invited questions and provided answers. I believe that the participant fully understands the implications and voluntary nature of the study.

(Signature of Investigator) (Date)

Phone Number

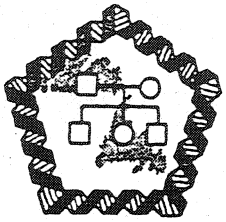
Assent of minor participant (if appropriate)

(Signature of Minor Participant) (Age ____)

Relationship to Participant Named Above

Newfoundland and Labrador Medical Genetics Program

Information and Appointments
Janeway Genetics Clinic (709) 778-4363
Health Science Centre Genetics Clinic (709) 737-6807
Outreach Program Genetics Clinic
Central Region Phone (709) 651-3306
Western Region Phone (709) 637-5253



Janeway Child Health Centre
Janeway Place
St. John's, NF
A1A 1R8

DNA Consent Form

I agree that a sample of blood may be drawn on _____ for genetic testing and/or banking and that the DNA obtained from the sample will be stored in the NLMGP Molecular Laboratory.

I understand that:

1. The sample will involve giving 10-15 mls of blood for preparation of the DNA.
2. This process is ***voluntary*** and I may withdraw my consent at any time without penalty.
3. ***If there is no test currently available*** for the condition of concern in my family, the sample will be stored as DNA. This does not guarantee a test will be available in future. Banking this sample may be of no direct benefit to my family or me.
4. ***If a test becomes available in the future***, the NLMGP may contact me to discuss whether further DNA testing is appropriate.
5. ***If a test is currently available*** for the condition of concern in my family, the possible results will be discussed with me. This includes the possibility that the testing may be inconclusive and a result will not be available.
6. The ***test results*** will be strictly confidential. They may, however, be used anonymously to help interpret test results for other members of my family.
7. These tests can sometimes point out ***discrepancies in parentage***. Such information will be kept confidential and will not be released even to family members directly involved.
8. The ***accuracy*** of any result is based on the current knowledge of the disease gene in question. Due to limitations in our understanding, human error, or technical difficulties, there is a small chance that a result could prove to be incorrect.
9. It is my responsibility to keep the NLMGP informed of any ***change of address*** so that they will be able to contact me in future if the need arises.

My signature on this form indicates that I have read the information on the previous page and understand that: (tick one)

☐ Direct DNA analysis will be done on this sample to test for _____

☐ Linkage analysis comparing this sample to others in my family will be done to test for _____

☐ No test is available at this time and the sample will be stored as DNA in the NLMGP Molecular Diagnostic Laboratory. but the DNA will be studied to identify any changes in colon cancer genes.

Future use of this DNA sample: (tick all that apply)

☐ I would like the DNA sample destroyed following completion of the above test.

☐ I would like the DNA to remain banked for the future use of other family members.

☐ I agree to the anonymous use of this DNA for any future research approved by a Research Ethics Board

☐ This DNA may be used at any time without restriction.

_____ _____

Patient's signature Date

_____ / _____ _____

Signature of legal guardian / Relationship to patient Date

_____ _____

Signature of Witness Date

...ical Genetics Program

...nd Appointments:
...netics Clinic (709) 778-4363
...ence Centre Genetics Clinic (709) 737-6807
...atellite Genetics Clinic (709) 651-3306
...Satellite Genetics Clinic (709) 637-2260

Janeway Child Health Centre
Janeway Place
St. John's, NF
A1A 1R8

Authorization for Release of Medical Information

** Please return a copy of this form with the medical record**

Medical Records Requested:
- ✓ Clinical Diagnostic Summaries
- ___ Birth Record
- ✓ Consultants Reports
- ✓ Laboratory reports
- ✓ Diagnostic Imaging reports
- ✓ Operative and/or Pathology reports
- ___ Other: _____

I, _____ hereby consent to the release of information to the Medical Genetics Program c/o: Medical Genetics Program, Janeway Child Health Centre, Janeway Place, St. John's, Nfld., A1A 1R8, from the medical records of:

NAME: _____ MAIDEN NAME: _____

ADDRESS: _____ PARENTS/SPOUSE _____

DATE OF BIRTH: _____ DATE OF DEATH: _____

MCP#: _____ CHART# _____

DATE OF HOSPITALIZATION: _____

NAME / ADDRESS OF HOSPITAL: _____

_____ _____ _____

SIGNATURE RELATIONSHIP DATE

****

Medical Genetics program Use: NLMGP# _____

Patient Name: _____

Geneticist / Genetic Counsellor: _____

FAX (709) 778-4190
cience Centre FAX (709) 737-3374

Outreach FAXES - Central (709) 651-3341
Western (709) 637-2616

APPENDIX F: Medical Chart Extraction form

Colorectal Chart Extraction Form

Date_____

ID # _____ MCP #_____

DOB_____

Address_____

_____

Telephone #_____

Sex _____

Physician_____

Attending Physician_____

Centre Physician_____

Diagnosis_____

Date of Diagnosis_____ Age at Diagnosis_____

Diagnosing Hospital_____

Means of Diagnosis_____

_____

Presenting Symptoms_____

_____

Site Code_____ Behaviour Code_____

Topology Code_____ Histology Code_____

Grade_____

Staging method_____ Stage revision_____

Clinical Stage_____ Pathological Stage_____

Presence of Polyps Y/N if yes, # _____ Location _____

_____

Metastases Y/N if yes, Location _____

_____

Previous Cancer Y/N if yes, Type_____

_____

Age and year at Diagnosis_____

Treatment: surgery Y/N Type_____

Date_____

Hospital_____

Chemo Y/N Type and Duration_____

_____

Radiation Y/N Type and Duration_____

_____

Patient Status_____

Death Date_____

Family History_____

_____

_____

_____

_____

Comments_____

_____

_____

APPENDIX G: Microsatellite analysis methods and materials

DNA EXTRACTION USING THE QIAGEN DNeasy KIT

(5 x 10μ paraffin sections)

~ Dewax paraffin tissue with 1ml of toluene, let sit 5 - 10 min
- centrifuge 5 min, then decant toluene
- repeat with toluene, centrifuge and decant
- clear with 1ml absolute alcohol, let sit 5 - 10 min
- centrifuge 5 min, then decant alcohol
- repeat with alcohol, centrifuge and decant
- rinse with 1ml PBS, let sit 5 - 10 min
- centrifuge 5 min, then decant PBS
- repeat with PBS, centrifuge and decant
- air dry tissue at room temp for 10 - 15 min

~ Digestion at 55°overnight (or until tissue digested). Add to each sample:
- 180μl ATL buffer
- 20μl proteinase K, then mix by vortexing, and incubate at 55°for a couple of hours to overnight.
  (If tissue is not all digested, add more proteinase K.)

~ Vortex for 15 sec. Add 200μl Buffer AL to the sample, mix thoroughly by vortexing, and incubate at 70°C for 10min.

~ Add 200μl absolute ethanol to the sample, and mix thoroughly by vortexing. (A white ppt may form, you must get all of it into the minicolumn)

~ Pipette the mixture into the DNeasy minicolumn sitting in a 2ml collection tube (provided). Centrifuge for 1 min at ≥6000g (8000rpm). Discard flow-through and collection tube.

~ Place the DNeasy mini column in a new 2ml collection tube (provided), add 500μl Buffer AW1, and centrifuge for 1 min at ≥6000g (8000rpm). Discard flow-through and collection tube.

~ Place the DNeasy mini column in a 2ml collection tube (provided), add 500μl Buffer AW2, and centrifuge for 3 min at full speed to dry the DNeasy membrane. Discard flow-through and collection tube.

~ Place the DNeasy mini column in a clean 1.5ml microcentrifuge tube (not provided), and pipette 100μl Buffer AE directly onto the DNeasy membrane. Incubate at room temp for 1 min, and then centrifuge for 1 min at ≥6000g (8000rpm) to elute.

DNA EXTRACTION BY SALTING OUT

(5 x 10μ PARAFFIN SECTIONS)

~ Dewax paraffin tissue with 1ml of toluene, let sit 5 - 10 min
- centrifuge 5 min, then decant toluene
- repeat with toluene, centrifuge and decant
- clear with 1ml absolute alcohol, let sit 5 - 10 min
- centrifuge 5 min, then decant alcohol
- repeat with alcohol, centrifuge and decant
- rinse with 1ml PBS, let sit 5 - 10 min
- centrifuge 5 min, then decant PBS
- repeat with PBS, centrifuge and decant
- air dry tissue at room temp for 10 - 15 min

~ Digestion at 55°overnight (or until tissue digested). Add to each sample:
- 425μl of 0.5x TBE buffer
- 50μl of 10% SDS
- 25μl of proteinase K (0.5μg/μl)

(If tissue is not all digested, add more proteinase K.)

~ Add 135μl (0.27 volume) of saturated 6M NaCl (salting out protein). Mix well!

~ Centrifuge for 5 min at 2000 rpm

~ Transfer supernatant into similar tubes (discard original tube with pellet)
- left with a volume ~ 600μl

~ Add 1.5ml of cold absolute alcohol (~2.5x volume). (Just fill to top of tube)

The DNA should suspend as strands near the top of the tube. If it doesn't:
- add 12.5μl 3M sodium acetate and put in the freezer for a few minutes
- centrifuge (cold) for 20 min, decant supernatant
- wash pellet once with 70% ethanol
- air dry pellet for a few min
- dissolve pellet in 20 - 30 μl TE buffer

Buffers

PBS

8g NaCl
0.2g KCl
1.44g Na_2HPO_4 (sodium phosphate)
0.24g KH_2PO_4 (potassium dihydrogen orthophosphate)

Add above (in that order) to 800ml dH_20, dissolve by magnetic stirrer. Adjust pH to 7.4 with HCl. Add dH_2O to bring to 1 litre.

5x TBE

54g	Tris Base
27.5g	boric acid
20ml	0.5M EDTA

make up to 1 litre with dH_2O
(Note: dilute with dH_2O to make 0.5x TBE for this protocol)

TE

10mM Tris.Cl (pH 8.0)
1mM EDTA (pH 8.0)

10ml of 1M Tris.Cl stock and 2ml of 0.5M EDTA stock made up to 1 litre with dH_20.

Reagents

6M NaCl (saturated)

350.6g NaCl made up to 1 litre with dH_20

5M is saturated, but make up 6M to be sure. Leave on the magnetic stirrer overnight to be sure to dissolve as much as possible.

10% SDS

10g SDS

Make up to 1 litre with dH_20. (Carefully! Can easily form a lot of bubbles.)

3M sodium acetate

246.1g sodium acetate

Make up to 1 litre with dH_20

(Or 12.3g made up to 50ml)

Proteinase K (0.5μg/μl)

0.01g proteinase K

Make up to 20ml with dH_20.

DNA EXTRACTION FROM WHOLE BLOOD

(for 5 - 7ml blood collected in EDTA tubes)

~ Transfer blood to 50ml centifuge tubes.

~ Add 5 volumes of warm (37°C) red cell lysis buffer to 1 volume of blood. Incubate at 37°C for 5 min.

~ Centrifuge at 2500 rpm for 5 min.

~ Pour off supernatant leaving white cell pellet at bottom.

~ Add 10ml saline. Vortex and centrifuge for 5 min again.

~ Pour off supernatant. Add 3ml nuclei lysis buffer to the cell pellet. Vortex and transfer to a 15ml centrifuge tube.

~ Add 0.2ml of 10% SDS and 0.5ml of pronase E solution.

~ Incubate at 37°C overnight (or at 55°C for 2 hours).

~ Add 1ml saturated NaCl, shake vigorously for 15 sec, and centrifuge at 2500 rpm for 15 min.

~ Gently pour supernatant into a 15ml tube and add 2 volumes of absolute ethanol; invert the tube several times to ppt the DNA.

~ Fish out the DNA clump with a 9" glass hook (9" Pasteur pipette; melt tip to form a hook). Wash several times with a stream of 70% ethanol. Let air dry.

~ Dissolve DNA in 300 - 500μl TE overnight. Gently mix sample on a rotator for an hour or so.

Buffers

Red Cell Lysis Buffer (1L)

900ml 0.155M NH_4Cl
100ml 0.17M Tris.HCl

To make 0.155M NH_4Cl:

Since 53.49g/mol, need 53.49g/L for 1M
Need 0.155M, therefore 53.49 x 0.155 = 8.29g/L

To make 0.17M Tris.HCl:

Since 157.6g/mol, need 157.6g/L for 1M
Need 0.17M, therefore 157.6 x 0.17 = 26.8g/L

Nuclei Lysis Buffer (1L)

10mM Tris.HCl
400mM NaCl
2mM EDTA

Tris.HCl: 157.6g/L = 1000mM
10mM = 1.576g/L Tris.HCl

NaCl: 58.44g/L = 1000mM
400mM = 23.38g/L NaCl

EDTA: 4ml of stock 0.5M EDTA

TE Buffer (1L)

10mM Tris.Cl (pH 8.0)
1mM EDTA (pH 8.0)

Tris.Cl: 10ml of stock 1M Tris.Cl

EDTA: 2ml of stock 0.5M EDTA

Reagents

6M NaCl (saturated)

350.6g NaCl made up to 1 litre with dH_20

5M is saturated, but make up 6M to be sure. Leave on the magnetic stirrer overnight to be sure to dissolve as much as possible.

Saline (0.85% NaCl)

8.5g NaCl

Make up to 1 litre with dH_20.

10% SDS

10g SDS

Make up to 1 litre with dH_20. (Carefully! Can easily form a lot of bubbles.)

Protease working solution (7mg/ml in 1% SDS and 2mM EDTA)

To make up 50ml:

350mg protease

5ml 10% SDS

200μl 0.5M EDTA

Make up to 50ml with dH_20. Invert (gently!!!) several times to mix.

Store at -20°C in 10ml aliquots.

Proteinase K working solution (3mg/ml in 1% SDS and 2mM EDTA)

To make up 50ml:

150mg proteinase K

5ml 10% SDS

200μl 0.5M EDTA

Make up to 50ml with dH_20. Invert (gently!!!) several times to mix.

Store at -20°C in 10ml aliquots.

Polymerase Chain Reaction (PCR)

Final concentrations of reagents:

10x buffer ⇒ 1x
5x Q solution (optional) ⇒ 1x
dNTPs ⇒ 0.2mM each
$MgCl_2$ ⇒ 1.0 - 4.0mM (usually 1.5mM)
primers ⇒ as per directions
(ex. from Research Genetics, 0.6μM)
Taq ⇒ 1 unit per reaction up to 40-50μl (2.5 units per 100μl reaction)

example:

For 22 reactions of 25μl each, = 550μl volume.
Allow for error = 625μl total volume. (Equiv. of 25 rxns)
625μl - 25μl DNA = 600μl cocktail

301.25μl	dH_2O
62.5μl	10x buffer (1x)
125μl	5x Q solution (1x)
31.25μl	4mM dNTPs (0.2mM)
37.5μl	25mM $MgCl_2$ (1.5mM)
18.75μl	20μM F-primer (0.6μM)
18.75μl	20μM R-primer (0.6μM)
5μl	5 U/μl Taq (1U/reaction)

Reagents

4mM dNTPs (to make 500μl of stock)

20μl 100mM dGTP
20μl 100mM dATP
20μl 100mM dTTP
20μl 100mM dCTP
420μl dH_2O

Mix together, and store in aliquots at -20°C.

Thermocycler Conditions

For HotStar Taq from Qiagen, with primers from Research Genetics:

95°C for 15 min

36 cycles of: 94°C for 45 sec
57°C for 45 sec
72°C for 1 min

72°C for 7 min

soak at 4°C

Agarose Gel

For 0.75% gel (for DNA smear):
0.375g agarose in 50ml 0.5x TBE

For 2% gel (for PCR products):
1.0g agarose in 50ml 0.5x TBE

Use 0.5x TBE for running buffer.
The 0.5x TBE used for both gel and running buffer should have 0.5µg/ml ethidium bromide, so the gel doesn't need to be stained after it is run.

Loading Dye

Bromophenol blue
0.025g bromophenol blue (0.25%)
4g sucrose (40%)
Make up to 10ml with dH_20.

Bromophenol blue and xylene cyanol FF
0.025g bromophenol blue (0.25%)
0.025g xylene cyanol FF (0.25%)
3.0ml glycerol (30%)
Make up to 10ml with dH_20.

Acrylamide Gel

6% acrylamide, 7M urea gel solution

189g	urea (7M)
45ml	5x TBE (0.5x)
67.5ml	40% acrylamide:bis 19:1 (6%)

Make up to 450ml with dH_20.
Filter and degas on vacuum suction for about 15 minutes.
Store at 4°C in a dark bottle.

For a small gel (20 x 40cm):

No need for a plug.
For the gel:

75ml	gel solution
500µl	10% ammoniuim persulfate
50µl	TEMED

Prerun at 65W, run gel at 50W.

For a large gel (40 x 60cm):

For the plug:

30ml	gel solution
175µl	10% ammonium persulfate
175µl	TEMED

For the gel:

120ml	gel solution
1ml	10% ammonium persulfate
100µl	TEMED

Prerun at 150W, run at 110W.

General Rules:
2.5µl 10% ammonium persulfate per ml acrylamide
1µl TEMED per ml acrylamide

Buffers and Reagents

5x TBE

54g	Tris Base
27.5g	boric acid
20ml	0.5M EDTA

Make up to 1 litre with dH_2O.

10% ammonium persulfate

Use 0.1g ammonium persulfate per ml of dH_2O.
Weigh APS directly into a 1.5ml tube, then add appropriate amount of dH_2O.
Vortex to dissolve.
(Note: If not using fresh, store in aliquots at -20°C.)

Loading Dye

10ml	formamide (98%)
200µl	0.5M EDTA
2.5mg	xylene cyanol FF (0.025%)
2.5mg	bromphenol blue (0.025%)

Add directly to a 15ml centrifuge tube.
Spin on an electric rotator for awhile to mix thoroughly.
Store in aliquots at -20°C.

DNA Ladder

4µl	100bp ladder
11µl	dH_2O
15µl	loading dye

Store at 4°C.

APPENDIX H: Immunohistochemistry analysis methods and materials

Silver Staining Procedure

Plate preparation:

~Wipe a few drops of Rain Away onto the plate with the buffer chamber, allow to dry ~5 min, then rinse with a paper towel saturated with dH_2O.

~Add 3μl Bind Silane to 1ml 0.5% acetic acid in 95% ethanol in a fume hood. Invert to mix, then wipe over the entire surface of the flat plate with a Kim Wipe. Allow to dry ~5 min, then rinse 3 or 4 times with absolute ethanol.

Assemble gel apparatus and run.

Take down gel, and stain:

Tray	Solution	Time
1	fix/stop	20 min
2	dH_2O	2 min
3	dH_2O	2 min
2	dH_2O	2 min
3	silver stain	30 min
2	dH_2O	10 sec
1	developer	~5 min (until proper exposure)
1	fix/stop	5 min (add directly to developer solution)
2	dH_2O	2 min (no longer, bands will fade)
3	none	dry overnight

Solutions

0.5% acetic acid in 95% ethanol

Add 1ml of glacial acetic acid to 199ml of 95% ethanol.
Store at -20°C.

10mg/ml Sodium Thiosulfate

200mg sodium thiosulfate made up to 20ml with dH_2O.
Store at room temperature in 400µl aliquots.

Fix/stop Solution

1800ml	dH_2O
200ml	glacial acetic acid

Silver Stain Solution

2L	dH_2O
3ml	formaldehyde
2g	silver nitrate

Stir on magnetic stirrer to dissolve.
Store in a dark cupboard until ready to use.

Developer Solution

2L	dH_2O
3ml	formaldehyde
400µl	10mg/ml sodium thiosulfate
60g	sodium carbonate

Stir on magnetic stirrer to dissolve.
Store at 4°C until ready to use, to bring temperature of solution to between 4° and 10°C.
(Note: If colder than this, it will take longer for the gel to develop.)

0% Sodium Hydroxide

100g NaOH made up to 1L with dH_2O.
Store at room temperature.

MSH2 Immunohistochemistry

DAY 1

~ put a couple of inches of water in the pressure cooker, and put it on a hot plate set on high
~ meanwhile, put the slides:
- 5 min in toluene
- 5 min in toluene
- 10 dips in 100% alcohol
- 10 dips in 100% alcohol
- 10 dips in 95% alcohol
- 10 dips in 70% alcohol

~ run slides under tap water for about 5 min (to remove alcohol)
~ shake off excess water
~ put slides in 3% hydrogen peroxide for 10 min (to block endogenous peroxidases)
~ rinse in running water for about 5 min

Antigen Retrieval
~ add citrate buffer(pH 6.0) to the coplin jars (enough to cover the slides)
~ put the slides in the coplin jars and tighten the lids
~ put the coplin jars in the pressure cooker
~ heat on high until constant hissing, turn heat down to 5½ and leave for 9 min
~ turn off heat, gently lift pressure cooker to the bench
~ remove the black pressure valve, wait until the red valve drops, then remove lid
~ carefully take out the coplin jars and leave them on the bench for ~25 min to cool (make sure the lids are still on tight)

~ remove the slides from the coplin jars and lay them out on the humidifying chamber (as each row is laid out, spray with PBS to keep them from drying out)
~ one row at a time, stand the slides up and dry off the back and edges with gauze
~ put the slides back in the chamber, and add 2 to 4 drops of 5% goat serum in water onto each section, spread to fully cover each section
~ cover the humidifying chamber and leave for 20 min

Make up the Antibody
~ for MSH2 ⇒ 1 in 30 dilution (antibody kept at -20°C)
Dilute in 1% bovine serum albumin made up in PBS.
Need ~200µl per section
~ for 25 sections: 25 x 200µl = 5000µl
~ since 1 in 30 dilution: 5000 ÷ 30 = 167µl antibody
Mix 167µl antibody with 4833µl 1% BSA in PBS.

~ don't rinse off the goat serum!
~ stand the slides upright (one row at a time), and wipe the excess goat serum off the sides. Put the slides back in the chamber
~ drop 200µl (2 or 3 drops) of 1° antibody onto each section, spread to make sure each section is fully covered
~ put the cover back on the chamber, and put in the fridge for 24 hours

DAY 2

~ remove the humidifying chamber from the fridge
~ rinse each slide twice with PBS (spray above the section, so the tissue won't come off)
~ wipe the back and edges of the slide dry
~ add 200µl of Envision to each section, spread to make sure they are covered
~ cover the chamber and leave for 30 min

Make up DAB (1 tablet of DAB in 15ml PBS)
~ Need about 200µl per section, therefore, for 25 sections, need 5000µl. Make up a little extra, 5250µl.
~ for every 1ml of DAB, add 1µl 30% hydrogen peroxide
Therefore, 5250µl DAB and 5.25µl 30% H_2O_2

~ rinse the slides twice with PBS
~ one row at a time, dry back and edges
~ add 3 drops DAB + H_2O_2 per section, set timer for 7 min for that row
~ make sure it covers each section entirely
~ repeat with the other rows of slides (each row gets its own timer)
~ after 7 min, give one rinse with PBS
~ once all slides are done, rinse them in running tap water

~ shake off excess water
~ put them in hemotoxylin for 3 min
~ run in water for ~5 min
~ dip once in 1% acid alcohol (gets rid of excess hemotoxylin) ⇒ quickly get into water!
~ rinse in water 2-5 min
~ put in Scott's tap water until blue (about 1 min)
~ rinse in water for a couple of minutes
~ shake off excess water
~ dip slides 10 times in each:
    - 70% alcohol
    - 95% alcohol
    - 100% alcohol
    - 100% alcohol
    - toluene
    - toluene
~ leave in the final toluene
~ remove one slide at a time, put a drop of permount on the edge, and drop on a cover slip

MLH1 Immunohistochemistry

DAY 1

~ put a couple of inches of water in the pressure cooker, and put it on a hot plate set on high
~ meanwhile, put the slides:
- 5 min in toluene
- 5 min in toluene
- 10 dips in 100% alcohol
- 10 dips in 100% alcohol
- 10 dips in 95% alcohol
- 10 dips in 70% alcohol

~ run slides under tap water for about 5 min (to remove alcohol)
~ shake off excess water
~ put slides in 3% hydrogen peroxide for 10 min (to block endogenous peroxidases)
~ rinse in running water for about 5 min

Antigen Retrieval
~ add citrate buffer(pH 6.0) to the coplin jars (enough to cover the slides)
~ put the slides in the coplin jars and tighten the lids
~ put the coplin jars in the pressure cooker
~ heat on high until constant hissing, turn heat down to 5½ and leave for 9 min
~ turn off heat, gently lift pressure cooker to the bench
~ remove the black pressure valve, wait until the red valve drops, then remove lid
~ carefully take out the coplin jars and leave them on the bench for ~25 min to cool (make sure the lids are still on tight)

~ remove the slides from the coplin jars and lay them out on the humidifying chamber (as each row is laid out, spray with PBS to keep them from drying out)
~ one row at a time, stand the slides up and dry off the back and edges with gauze
~ put the slides back in the chamber, and add 2 to 4 drops of Universal Blocker onto each section, spread to fully cover each section
~ cover the humidifying chamber and leave for 20 min

Make up the Antibody
~ for MLH1 ⇒ 1 in 50 dilution (antibody kept at 4°C)
Dilute in 1% bovine serum albumin made up in PBS.
Need ~200µl per section
~ for 25 sections: 25 x 200µl = 5000µl
~ since 1 in 50 dilution: 5000 ÷ 50 = 100µl antibody
Mix 100µl antibody with 4900µl 1% BSA in PBS.

~ don't rinse off the Universal Blocker!
~ stand the slides upright (one row at a time), and wipe the excess Universal Blocker off the sides. Put the slides back in the chamber
~ drop 200µl (2 or 3 drops) of 1° antibody onto each section, spread to make sure each section is fully covered
~ put the cover back on the chamber, and put in the fridge for 24 hours

DAY 2

~ remove the humidifying chamber from the fridge
~ rinse each slide twice with PBS (spray above the section, so the tissue won't come off)
~ wipe the back and edges of the slide dry
~ add 200µl of Link Antibody (from the kit)* to each section, spread to make sure they are covered
~ cover the chamber and leave for 30 min
~ rinse the slides twice with PBS
~ add 200µl Streptavidin Peroxidase (from the kit)* to each section, spread to make sure they are covered
~ cover the chamber and leave for 30min

Make up DAB (from the kit)*
~ Need about 200µl per section, therefore, for 25 sections, need 5000µl. Make up a little extra.
~ for every 1ml from the big bottle, add 1 drop from the small bottle. Add both to the tube enclosed, and put the date on the cover (its good for one week). Shake to mix.

~ rinse the slides twice with PBS
~ one row at a time, dry back and edges
~ add 3 drops DAB per section, set timer for 7 min for that row
~ make sure it covers each section entirely
~ repeat with the other rows of slides (each row gets its own timer)
~ after 7 min, give one rinse with PBS
~ once all slides are done, rinse them in running tap water
~ shake off excess water
~ put them in hemotoxylin for 3 min
~ run in water for ~5 min
~ dip once in 1% acid alcohol (gets rid of excess hemotoxylin) ⇒ quickly get into water!
~ rinse in water 2-5 min
~ put in Scott's tap water until blue (about 1 min)
~ rinse in water for a couple of minutes
~ shake off excess water
~ dip slides 10 times in each:
    - 70% alcohol
    - 95% alcohol
    - 100% alcohol
    - 100% alcohol
    - toluene
    - toluene
~ leave in the final toluene
~ remove one slide at a time, put a drop of permount on the edge, and drop on a cover slip

* The kit is: DAKO LSAB + System, HRP

MSH6 Immunohistochemistry

DAY 1

~ put a couple of inches of water in the pressure cooker, and put it on a hot plate set on high
~ meanwhile, put the slides:
- 5 min in toluene
- 5 min in toluene
- 10 dips in 100% alcohol
- 10 dips in 100% alcohol
- 10 dips in 95% alcohol
- 10 dips in 70% alcohol

~ run slides under tap water for about 5 min (to remove alcohol)
~ shake off excess water
~ put slides in 3% hydrogen peroxide for 10 min (to block endogenous peroxidases)
~ rinse in running water for about 5 min

Antigen Retrieval
~ add citrate buffer(pH 6.0) to the coplin jars (enough to cover the slides)
~ put the slides in the coplin jars and tighten the lids
~ put the coplin jars in the pressure cooker
~ heat on high until constant hissing, turn heat down to 5½ and leave for 9 min
~ turn off heat, gently lift pressure cooker to the bench
~ remove the black pressure valve, wait until the red valve drops, then remove lid
~ carefully take out the coplin jars and leave them on the bench for ~25 min to cool (make sure the lids are still on tight)

~ remove the slides from the coplin jars and lay them out on the humidifying chamber (as each row is laid out, spray with PBS to keep them from drying out)
~ one row at a time, stand the slides up and dry off the back and edges with gauze
~ put the slides back in the chamber, and add 2 to 4 drops of Universal Blocker onto each section, spread to fully cover each section
~ cover the humidifying chamber and leave for 20 min

Make up the Antibody
~ for MSH6 ⇒ 1 in 75 dilution (antibody kept at 4°C)
Dilute in 1% bovine serum albumin made up in PBS.
Need ~200µl per section
~ for 25 sections: 25 x 200µl = 5000µl
~ since 1 in 75 dilution: 5000 ÷ 75 = 66.7µl antibody
Mix 66.7µl antibody with 4933.3µl 1% BSA in PBS.

~ don't rinse off the Universal Blocker!
~ stand the slides upright (one row at a time), and wipe the excess Universal Blocker off the sides. Put the slides back in the chamber
~ drop 200µl (2 or 3 drops) of 1° antibody onto each section, spread to make sure each section is fully covered
~ put the cover back on the chamber, and put in the fridge for 24 hours

DAY 2

~ remove the humidifying chamber from the fridge
~ rinse each slide twice with PBS (spray above the section, so the tissue won't come off)
~ wipe the back and edges of the slide dry
~ add 200µl of Link Antibody (from the kit)* to each section, spread to make sure they are covered
~ cover the chamber and leave for 30 min
~ rinse the slides twice with PBS
~ add 200µl Streptavidin Peroxidase (from the kit)* to each section, spread to make sure they are covered
~ cover the chamber and leave for 30min

Make up DAB (from the kit)*
~ Need about 200µl per section, therefore, for 25 sections, need 5000µl. Make up a little extra.
~ for every 1ml from the big bottle, add 1 drop from the small bottle. Add both to the tube enclosed, and put the date on the cover (its good for one week). Shake to mix.

~ rinse the slides twice with PBS
~ one row at a time, dry back and edges
~ add 3 drops DAB per section, set timer for 7 min for that row
~ make sure it covers each section entirely
~ repeat with the other rows of slides (each row gets its own timer)
~ after 7 min, give one rinse with PBS
~ once all slides are done, rinse them in running tap water
~ shake off excess water
~ put them in hemotoxylin for 3 min
~ run in water for ~5 min
~ dip once in 1% acid alcohol (gets rid of excess hemotoxylin) ⇒ quickly get into water!
~ rinse in water 2-5 min
~ put in Scott's tap water until blue (about 1 min)
~ rinse in water for a couple of minutes
~ shake off excess water
~ dip slides 10 times in each:
    - 70% alcohol
    - 95% alcohol
    - 100% alcohol
    - 100% alcohol
    - toluene
    - toluene
~ leave in the final toluene
~ remove one slide at a time, put a drop of permount on the edge, and drop on a cover slip

* The kit is: DAKO LSAB + System, HRP

Treating Slides with Histogrip

Treats 975 slides (13 boxes of 75).

~Dilute Histogrip: 10ml bottle into 500ml acetone

~Place glass slides into Histogrip solution for 2 minutes.

~After the 2 minutes, dip the slides 10 times in the same Histogrip solution.

~Rinse quickly in 3 changes (10 dips each) of distilled water.

~Dry slides in 60°C oven for about 1 hour.

~Store slides in a dust-proof box.

Note: Histogrip is from Zymed Laboratories Inc.

Immunohistochemistry Solutions

PBS (1L)

32g 8.0g sodium chloride
5.2g 1.3g disodium phosphate
16.0g 4.0g monosodium phosphate
4L Dissolve in dH_2O (bring to just under 1L). Adjust pH to 7.4 with 2M NaOH. Bring volume up to 1L with dH_2O. Store at room temp.

10mM Citrate Buffer (1L)

2.1g citric acid
Dissolve in dH_2O. Adjust pH to 6.0 with 2M NaOH. Bring volume up to 1L with dH_2O. Store at room temp.

2M NaOH (500ml)

40g NaOH
Dissolve in dH_2O and bring volume up to 500ml.

1% BSA in PBS (25ml)

0.25g bovine serum albumin (stored at 4°C)
Dissolve in 25ml PBS. Store at 4°C.
Note: make small volumes at a time, since crud seems to grow in this in the fridge in short periods of time.

3% Hydrogen Peroxide (1L)

30ml 30% H_2O_2
970ml dH_2O
Store at room temp.

Acid Alcohol (1%) (1L)

10ml conc. HCl (12M)
990ml 70% alcohol
Store at room temp.

Immunohistochemistry Solutions (cntd)

Scotts Tap Water (enough for 2L)

Mix half and half sodium bicarbonate and magnesium sulfate solutions, as follows:

7g sodium bicarbonate
Dissolve in 1L dH_2O.

40g magnesium sulfate
Dissolve in 1L dH_2O.

Note: Store separately, and only mix (50:50) when ready to use. Change the solution when it turns pink.

Hematoxylin (~2L)

~ Dissolve 180g of aluminum ammonium sulfate in 1200ml dH_2O.
~ Dissolve 12g hematoxylin in 75ml of 95% alcohol.
~ Combine the two solutions once each is dissolved.
~ Expose the mixture to light and air for one month in an unstoppered bottle (cover the top of the bottle with gauze).
~ After one month, filter and add 300ml glycerin and 300ml of 95% alcohol.
~ Allow to stand in light until colour is sufficiently dark.
~ Filter and keep in a tightly stoppered dark bottle at room temp.

HMSH2 PCR work table

Exon		primer	Size(bp)	PCR condition	PCR	Sscp gel	Glycerol gel	note
1	S1-F	Cgc att ttc ttc aac cag ga	279					contaminated
	S1-R	Cct ccc cag cac gcg cc						
1new	S1-S	Cgc att ttc ttc aac cag ga	279	55C, Mg:1.0mM	++.	*	*	
	S1-A	Cct ccc cag cac gcg cc						
2.1	S2-OF	Gtc cag cta ata cag tgc ttg	197	55C, Mg:1.5mM	++	*	*	
	S2-IR	Tcc ttg gat gcc tta ttt cc						
2.2	S2-IF	Ctg cag agt gtt gtg ctt agt a	202	55C, Mg:1.5mM	++	*	*	
	S2-OR	Cac att ttt att ttt cta ctc tta a						
2	S2-OF	Gtc cag cta ata cag tgc ttg	280	55C, Mg:1.5mM	++	++		
	S2-OR	Cac att ttt att ttt cta ctc tta a						
3	S3-F	Tat aaa att tta aag tat gtt caa g	389	55C, Mg:2mM	++	*	*	
	S3-R	Ttt cct agg cct gga atc tcc tct						
4	S4-F	Ttt ttg ctt ttc tta ttc ctt ttc	309	55C, Mg: 3mM	++	++	*	
	S4-R	Tga cag aaa tat cct tct aa						
5.1	S5-OF	Cca gtg gta tag aaa tct tcg	172	55C, Mg:2mM	++	*	*	
	S5-IR	Gac tgc tgc aat atc caa tt						
5.2	S5-IF	Cag atg att cca act ttg ga	180/181	55C, Mg:2mM	++	*	*	
	S5-OR	Agc ttc ttc agt ata tgt caa tg						
5	5of+or		277	55C, Mg: 3mM				
6	S6-F	Ttc act aat gag ctt gcc att c	246					Doesn't work
	S6-R	Gta taa tca tgt ggg taa c						
6new	S6-S	gtt ttc act aat gag ctt gcc	251	55C, Mg: 1.2mM	++	*	*	
	S6-A	gtg gta taa tca tgt ggg						
7	S7-F	Ctt acg tgc tta gtt gat aa	321	55C, Mg: 3mM	++	*	*	
	S7-R	Tat att gta tga gtt gaa gga aaa						
8	S8-F	Ttt gta ttc tgt aaa atg aga tct tt	217	55C, Mg: 3mM	++	+*	*	
	S8-R	Ctt tgc ttt tta aaa ata act act g						
9	S9-F	Ctt tac cca tta ttt ata gga tt	213	55C, Mg: 3mM	++	*	*	
	S9-R	Ata gac aaa aga att att cca ac						
10	S10-F	Tag tag gta ttt atg gaa tac ttt t	255	55C, Mg: 3mM	+++	*	*+43, *	
	S10-R	Tgt tag agc att tag gga att						
11	S11-F	Cat tgc ttc tag tac aca ttt	195	55C, Mg:1.5mM	++	*	* - 2, +2	
	S11-R	Cag gtg aca ttc aga aca tta						
12	S12-F	Tca gta ttc ctg tgt aca ttt	323	55C, Mg: 2mM	++	*	*	
	S12-R	Tta ccc cca caa agc cca a						
13	N16324	5`cgc gat taa tca tca gtg	348	55C, Mg:1.5mM	++	*	*	
	C16340	5`gga cag aga cat aca ttt cta tc						
14	S14-F	Cca cat ttt atg tga tgg gaa	349	55C, Mg:1.5mM	++	*	*	
	S14-R	Ggt agt aag ttt ccc att acc aag						
15	S15-F	Ctt ctc atg ctg tcc cct c	256	55C, Mg:1.2mM	++	*	*	
	S15-R	Gag aag cta agt taa act atg						
16	S16-F	Att act cat ggg aca ttc aca	225	55C, Mg:1.5mM	++	*	*	
	S16-R	Ctt cat tcc att act ggg at						

DAKO

INSTRUCTIONS

Large Volume DAKO LSAB®+ Kit, Peroxidase

Universal

K0690

These instructions apply to the **Universal Large Volume DAKO LSAB®+ Kit, Peroxidase** (L.V. DAKO LSAB®+ Kit, HRP) for use with primary antibodies from **RABBIT, MOUSE and GOAT** supplied by the user.

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1. INTENDED USE

FOR LABORATORY USE.

This kit, consisting of labelled streptavidin biotin (LSAB) reagents, is intended for the qualitative demonstration of antigens in paraffin-embedded tissues, cryostat tissues and cell preparations. Tissues processed in a variety of fixatives including ethanol, B-5, Bouin's, and neutral buffered formalin may be used.

2. SUMMARY AND EXPLANATION

The purpose of immunohistochemical (IHC) staining techniques is to allow for the visualization of tissue (cell) antigens. Originally this was accomplished by the direct technique using enzymes conjugated directly to an antibody with known antigenic specificity. Although this technique lacked the sensitivity of later methods, it allowed the direct visualization of tissue antigens using a standard light microscope.[1]

The sensitivity of IHC techniques was significantly improved with the development of an indirect method. In this two-step method several enzyme-labelled secondary antibodies reacted with the antigen-bound primary antibody. Subsequently the peroxidase-

antiperoxidase (PAP) methodology was introduced which used a three-step method consisting of the sequential application of primary antibody, link antibody and a PAP complex. This method rendered greater sensitivity than traditional two-step indirect techniques.[2] Finally, the strong affinity of avidin for biotin was exploited in the three-step avidin-biotin complex (ABC) method developed by Hsu et al.[3] This method provided a further increase in sensitivity over other existing methods.

The L.V. DAKO LSAB®+ Kit, HRP utilizes a refined avidin-biotin technique in which a biotinylated secondary antibody reacts with several peroxidase-conjugated streptavidin molecules.[4]

The L.V. DAKO LSAB®+ Kit, HRP is a sensitive and versatile IHC staining procedure which permits the simultaneous processing of numerous specimens in just over one hour. When compared to the ABC method, labelled avidin-biotin methods have been shown to provide an increase in sensitivity of up to eight times.[5-8] The L.V. DAKO LSAB®+ Kit, HRP is an extremely sensitive LSAB method, and, as a result, optimal dilutions of primary antibody are up to 20 times higher than those used for the more traditional PAP technique, and several fold greater than those used in traditional ABC or LSAB methods. This kit offers an enhanced signal generating system for the detection of antigens present in low concentrations, or for increased staining intensity in compensation for low titer primary antibodies. Primary antibodies produced in rabbit, mouse or goat are labelled by the biotinylated link antibody provided in this kit. The color reaction is developed using a substrate-chromogen solution, resulting in a colored precipitate at the antigen site.

3. PRINCIPLES OF PROCEDURE

The technique used in this kit is based on the LSAB method. Endogenous peroxidase activity can be quenched by first incubating the specimens for five minutes in 3% hydrogen peroxide (supplied by user). The specimens are then incubated with an appropriately characterized and diluted rabbit, mouse or goat primary antibody, followed by sequential incubations with biotinylated link antibody and peroxidase-labelled streptavidin. Staining is completed after incubation with substrate-chromogen solution (supplied by user). For optimal sensitivity DAKO® Large Volume DAB+ (Code No. K3467) is recommended for substrate-chromogen.

4. REAGENTS

The following materials are included in this kit:

Manual Staining Reagents

Quantity	*Description*
1x110 mL	**Link :** Biotinylated anti-rabbit, anti-mouse and anti-goat immunoglobulins in phosphate

	buffered saline (PBS), containing carrier protein and 15mM sodium azide.
1x110 mL	**Streptavidin Peroxidase:** Streptavidin conjugated to horseradish peroxidase in PBS containing carrier protein and anti-microbial agents.

*DAKO Autostainer Reagents**

10x11 mL	**Link :** Biotinylated anti-rabbit, anti-mouse and anti-goat immunoglobulins in phosphate buffered saline (PBS), containing carrier protein and 15mM sodium azide.
10x11 mL	**Streptavidin Peroxidase:** Streptavidin conjugated to horseradish peroxidase in PBS containing carrier protein and anti-microbial agents.

*DAKO Autostainer is available only in North America, South America, Australia and New Zealand.

MATERIALS REQUIRED BUT NOT SUPPLIED

Primary antibody and negative control reagent

Positive and negative control specimens

Xylene, toluene or xylene substitutes

Absolute ethanol

95% ethanol

3% hydrogen peroxide

Distilled water

Wash bottles

Wash solution, NOT containing sodium azide

Timer (2-30 minutes)

Absorbent wipes

Substrate-chromogen reagents, for optimal sensitivity DAKO® Large Volume DAB+ (Code No. K3468) is recommended due to its increased sensitivity; alternatively, DAKO® DAB Chromogen tablets (Code No. S3000), Large Volume DAKO® AEC Substrate System, Ready-to-use (Code No. K3464) or Large Volume DAKO® AEC Substrate System (Code No. K0696) can be used.

Staining jars

Counterstain

37mM ammonium hydroxide

Mounting media, such as DAKO® Faramount (Code No. S3025) or DAKO Glycergel® (Code No. C0563)

Coverslips

Standard light microscope (20-800x)

5. PRECAUTIONS

A. Product Specific

1. FOR LABORATORY USE.
2. Sodium azide which is used as a preservative is toxic if ingested. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal, flush with large volumes of water to prevent azide build-up in plumbing.[9,10]
3. Do not use reagents beyond the expiration date.
4. Do not substitute reagents from other lot numbers or from kits of other manufacturers.
5. Do not store kit components or perform staining in strong light, such as direct sunlight.

B. General

1. Specimens, before and after fixation, and all materials exposed to them, should be handled as if capable of transmitting infection and disposed of with proper precautions.[11]
2. Inhalation or ingestion of the highly allergenic fixative formaldehyde is harmful. Prepare in hood. If swallowed, induce vomiting. If skin or eye contact occurs, wash thoroughly with water.
3. Organic reagents are flammable. Do not use near open flame.
4. Never pipette reagents by mouth and avoid their contact with skin and mucous membranes.
5. Avoid microbial contamination of reagents as incorrect results may occur.
6. Avoid splashing of reagents or generation of aerosols.

6. REAGENT PREPARATION

A. Wash Solution

PBS, 0.05M Tris-HCl buffer or Tris Buffered Saline (DAKO® PBS, Code No. S3024, DAKO® TBS, Code No. S3001), pH 7.2-7.6, *not containing sodium azide* are suitable wash solutions. Distilled water may be used for rinsing off the hydrogen peroxide (Section 9.B. Step 1), substrate-chromogen solution, and counterstain. Unused wash solution may be stored at 2-8°C. Discard if solution becomes cloudy.

B. Primary Antibody and Negative Control Reagent

DAKO LSAB® Ready-to-use Primary Antibodies and Negative Controls (N-series) are *not* recommended for use with this kit. However, DAKO offers concentrated monoclonal and polyclonal antibodies suitable for use in IHC. Due to the high sensitivity of the L.V. DAKO LSAB®+ Kit, HRP, primary antibody dilutions may range from five- to twenty-fold greater than those used in traditional IHC methods. Optimal dilutions must be determined experimentally by the user. Dilutions should be prepared using 0.05M Tris-HCl buffer, pH 7.2-7.6, containing 1% bovine serum albumin or DAKO® Antibody Diluent (Code No. S0809). For primary antibodies an incubation time of 30 minutes is recommended.

C. Substrate-Chromogen Solution

DAKO® Large Volume DAB+ (Code No. K3468) is recommended for use with the L.V. DAKO LSAB®+ Kit, HRP due to its increased sensitivity. Alternatively, the DAKO® DAB Chromogen tablets (Code No. S3000), the Large Volume DAKO® AEC Substrate System, Ready-to-use (Code No. K3464) or the Large Volume DAKO® AEC Substrate System (Code No. K0696) can be used. Please follow the instructions provided with each substrate system for substrate-chromogen preparation.

D. Counterstain

DAB chromogen yields an alcohol insoluble end-product and can be used with alcohol-based hematoxylin. The colored end-product of the AEC substrate-chromogen reaction is alcohol soluble and should only be used with aqueous-based counterstains such as Mayer's hematoxylin. When using hematoxylin, counterstaining is completed by immersing tissue sections into 37mM ammonia water. Ammonia water is prepared by mixing 2.5 mL 15M (concentrated) ammonium hydroxide with 1 liter water. Unused ammonia water may be stored at room temperature in a tightly capped bottle.

E. Mounting Media

DAKO® Faramount, Aqueous Mounting Medium, Ready-to-use (Code No. S3025) or DAKO Glycergel® Mounting Medium (Code No. C0563) is recommended for aqueous mounting. Liquify DAKO Glycergel® by warming to approximately 40 ± 5°C prior to use. A nonaqueous mounting medium can be used with DAB chromogen.

7. STORAGE AND HANDLING

Reagents of the L.V. DAKO LSAB®+ Kit, HRP are to be stored at 2-8°C. Do not freeze.

8. SPECIMEN COLLECTION AND PREPARATION

Specimens processed in a variety of fixatives may be used. The choice of fixative and method is best made by the user within the context of their own laboratory and institutional constraints. For recommended methods and techniques of specimen fixation, please refer to *Histological and Histochemical Methods: Theory and Practice.*[12]

Survival of tissue antigens for immunological staining may depend on the type and concentration of fixative, on fixation time, and on the size of the tissue specimen to be fixed.[13] It is thus important to maintain optimal, standardized fixation conditions whenever possible in order to obtain reproducible staining. Where possible, the use of thinner specimens coupled with shorter fixation times is recommended. Prolonged exposure to fixatives may result in the masking of antigens and hence contribute to reduced staining. Zenker's fluid, B-5, and Bouin's have often been recommended as milder fixatives for paraffin-embedded specimens.[14,15]

A. Paraffin-Embedded Tissue

Tissue Fixation in Formaldehyde-Based Solutions

For optimal fixation in neutral buffered formalin, tissue should be cut in blocks of approximately 1.0 x 1.0 x 0.5 cm, and immediately placed in 5-10 mL formalin per block. The tissue should not remain in formalin for more than 24 hours or more than three hours in B-5.

Please refer to the manufacturer's fixation protocol supplied with the fixing reagents for proper procedures. Processing may be completed using an automatic tissue processor, graded alcohols, xylene or xylene substitute, and paraffin wax. Temperatures greater than 60°C MUST be avoided. Rapid high temperature processing destroys antigenicity.

Tissues fixed in mercuric chloride-containing fixatives (e.g. Zenker's, B-5, etc.) should be "de-Zenkerized" prior to application of the primary antibody.

Formaldehyde is known to induce steric changes in the antigen molecules by forming intermolecular cross-linkages. Excessive formalin fixation can mask antigenic sites and diminish specific staining. Enzymatic digestion of tissue sections prior to incubation with primary antibody may recover some of these sites. Deparaffinized and rehydrated tissue sections can be digested for six minutes at room temperature in a solution containing 0.025% protease Type XXIV and 0.025% $CaCl_2$, in Tris-HCl buffer, pH 7.2-7.6. The use of coated slides is recommended for greater adherence of tissue sections to the glass slides.

Rinse thoroughly with distilled water and continue with the staining procedure as outlined in Section 9.B. Step 1. Other proteolytic enzymes, such as proteinase K (DAKO® Proteinase K, Code No. S3004 or DAKO® Proteinase K, Ready-to-use, Code No. S3020), pepsin (DAKO® Pepsin, Code No. S3002), or trypsin can also be used.

Tissue Fixation in Ethanol

The following procedure for fixation in ethanol is recommended: immerse the tissue blocks in absolute ethanol for 48 hours at room temperature, followed by two 1-hour baths in fresh xylene and two 1-hour baths in liquid paraffin. The tissue may then be embedded.

Adherence of Tissue Sections to Slides

Poly-L-lysine coated or DAKO® Silanized Slides (Code No. S3003) are recommended for proper adherence of tissue sections during staining procedures. The use of coated slides is especially recommended when enzymatic predigestion is performed. The tissue sections should be dried onto the slides at temperatures not to exceed 37°C.

Deparaffinization and Rehydration

The embedding medium must be completely removed from the specimen. Any residual medium can cause an increase in background and obscure specific staining. Xylene and alcohol solutions should be changed after every 40 slides. Toluene or xylene substitute such as Histoclear may be used in place of xylene.

1. Place slides in xylene bath and incubate for five minutes. Repeat once.
2. Tap off excess liquid and place slides in absolute ethanol for three minutes. Repeat once.
3. Tap off excess liquid and place slides in 95% ethanol for three minutes. Repeat once.
4. Tap off excess liquid and place slides in distilled or deionized water for 30 seconds. Unless proteolytic predigestion is required, commence staining procedure as outlined in Section 9.B. Step 1.

B. Frozen Tissue

Cryostat sections (five to eight microns) should be cut from snap-frozen tissue blocks (approximately 1.0 x 1.0 x 0.5 cm) and air-dried for 2-24 hours. Dried sections are ready for immediate processing or may be wrapped air-tight and stored frozen at -20°C or lower.

If stored frozen, sections should be brought to room temperature before unwrapping. Tissue sections may be fixed in acetone for 10 minutes. Alternatively, fixation may be performed after sectioning and air-drying, prior to frozen storage. Allow sections to air-dry after fixation. Submerge slides in buffer bath for five minutes, then commence staining procedure as outlined in Section 9.B., Step 2.

9. STAINING PROCEDURE

A. Procedural Notes

Before using this kit for the first time the user should read the instructions carefully and become familiar with the contents. The colors of solutions are keyed to the condensed instructions on the inside cover of the kit box. See Section 5 for precaution information.

The reagents and instructions supplied in this kit have been designed for optimal performance. Further dilution of the kit reagents or alteration of incubation times or temperatures may give erroneous results.

All kit reagents should be equilibrated to room temperature prior to performing the procedure; likewise, all incubations should be performed at room temperature.

Do not allow tissue sections to dry during the staining procedure. Provided that the workplace is not exposed to drafts, the slides need not be covered during the recommended incubation times. However, if incubations are prolonged, cover or place slides in a humid environment.

If the staining protocol must be interrupted, slides may be kept in the buffer bath which follows the link incubation (Step 3) for up to one hour without affecting staining performance.

B. Staining Protocol

STEP 1 HYDROGEN PEROXIDE

Tap off excess water and carefully wipe around specimen.

Apply enough user supplied 3% hydrogen peroxide to cover specimen.

Incubate five minutes.

Rinse *gently* with distilled water or wash solution from a wash bottle and place in fresh buffer bath.

STEP 2 PRIMARY ANTIBODY AND NEGATIVE CONTROL REAGENT

Tap off excess buffer and wipe slide as before.

Apply enough user prepared primary antibody or negative control reagent to cover specimen.

Incubate 30 minutes unless otherwise specified.

Rinse *gently* with wash solution from a wash bottle and place in buffer bath.

STEP 3 LINK

Immediately tap off excess buffer and wipe slide as before.

Apply enough YELLOW drops of Link to cover specimen.

Incubate 15 minutes. For further enhancement of sensitivity incubate for 30 minutes.

Rinse slide as in Step 2.

STEP 4 STREPTAVIDIN PEROXIDASE

Wipe slide as before.

Apply enough RED drops of Streptavidin to cover specimen.

Incubate 15 minutes. For further enhancement of sensitivity incubate for 30 minutes.

Rinse slide as before.

STEP 5 SUBSTRATE-CHROMOGEN SOLUTION

Wipe slide as before.

Apply enough of the user prepared substrate-chromogen solution to cover specimen.

Incubate as recommended in instructions provided with substrate-chromogen.

Rinse *gently* with distilled water from wash bottle.

STEP 6 COUNTERSTAIN (optional)

Cover specimens with hematoxylin or place slides in a bath of hematoxylin (see Section 6.D.). Incubate for two to five minutes, depending on the strength of the hematoxylin used.

Rinse *gently* with distilled water from a wash bottle.

Dip 10 times into a wash bath filled with ammonia water (see Section 6.D.).

Place in distilled or deionized water for two minutes.

STEP 7 MOUNTING

Specimen may be mounted and coverslipped with an aqueous-based mounting medium such as DAKO® Faramount (Code No. S3025) or DAKO Glycergel® (Code No. C0563). A nonaqueous mounting medium may be used with DAB chromogen.

Note: The AEC reaction product is soluble in organic solvents and therefore not compatible with toluene- or xylene-based, permanent mounting media.

10. QUALITY CONTROL

A. Positive Control Specimen

In order to ascertain that all kit reagents are functioning properly, a positive control specimen should accompany each staining run. It should be noted that the known positive control specimen should *only* be utilized for monitoring the accurate performance of the kit reagents. If positive control specimens fail to demonstrate positive staining, labelling of test specimens should be considered invalid.

B. Negative Control Specimen

A negative control specimen stained with primary antibody should be used with each staining run to verify the specificity of the primary antibody. If staining occurs in the negative control specimen, results with the test specimen should be considered invalid.

C. Negative Control Reagent

A negative control reagent, allowing the recognition of nonspecific staining should be used with each specimen. This will allow improved interpretation of specific staining at the antigen site. Alternatively, an antibody not specific for any specimen antigen, diluted in Tris-HCl buffer, pH 7.2-7.6, containing 1% bovine serum albumin or DAKO® Antibody Diluent (Code No. S0809) may be used.

For other suggested procedures to be used in quality control assessment, please see references 16 and 17.

11. INTERPRETATION OF STAINING

Examine the positive control specimen for the presence of a colored end-product at the site of the target antigen. DAB chromogen yields a characteristic brown end-product whereas AEC chromogen yields a red end-product. The presence of these colors can be interpreted as a positive staining result, indicating proper performance of kit reagents. The absence of specific staining in the negative control specimen confirms the specificity of the primary antibody.

Examination for any nonspecific staining present on the negative control reagent slide is recommended next. Nonspecific staining, if present, is of rather diffuse appearance and is frequently observed in connective tissue.

Test specimens stained with primary antibody should then be examined. Positive staining intensity should be assessed within the context of any background staining of the negative control reagent. The presence of a colored end-product can be interpreted as a positive staining result. The absence of a staining reaction can be interpreted as a negative staining result.

Use only intact cells for interpretation since necrotic or degenerated cells often stain nonspecifically. Precipitates may form if, for example, specimens are allowed to dry during the staining procedure.

This may be apparent at the edge of the specimen. Use of low magnification for scanning will minimize this potential misinterpretation.

Depending on the length of the incubation time in hematoxylin, counterstaining will result in pale to dark blue coloration of cell nuclei.

12. LIMITATIONS

Tissue staining is dependent on the handling and processing of the tissue prior to staining. Improper fixation, freezing, thawing, washing, drying, heating, or sectioning may produce artifacts or false-negative results.

Results will not be optimal if old or unbuffered fixatives are used, or if excessive heat is used during embedding or during attachment of sections to slides.

False-positive results may be seen due to nonspecific binding of proteins. Although DAKO LSAB®+ Kits do not require the use of a separate blocking reagent, in some cases the application of a blocking reagent containing an irrelevant protein, prior to incubation with the primary antibody may be useful for reducing background. A recommended blocking reagent may be DAKO® Protein Block Serum-Free (Code No. X0909), or a blocking solution can be made from normal swine serum such as DAKO® Serum Normal, Swine (Code No. X0901) diluted to 20% in 0.05M Tris-HCl buffer, pH 7.2-7.6.

Endogenous avidin-binding activity (EABA) has been noted in frozen sections of liver (entire hepatic nodule) and kidney (tubular epithelium), as well as in frozen and formalin-fixed lymphoid tissue (paracortical histiocytes).[18,19] EABA can be suppressed by sequential 10-minute incubations, first with 0.1% avidin and then with 0.01% biotin in 0.05M Tris-HCl buffer, pH 7.2-7.6, prior to Section 9.B. Step 1.

Endogenous peroxidase or pseudoperoxidase activity can be found in hemoproteins such as hemoglobin, myoglobin, cytochrome, and catalase as well as in eosinophils.[20,21] In formalin-fixed tissue this activity can be inhibited by incubating the tissue in 3% hydrogen peroxide for five minutes prior to the application of primary antibody. Blood and bone marrow smears can be treated with DAKO® Peroxidase Blocking Reagent (Code No. S2001), however, this procedure does not abolish the reddish-brown pigment of hemoproteins. A solution of methanol-hydrogen peroxide can also be used, however, some antigens may become denatured with this procedure.

Necrotic or degenerated cells will offer stain nonspecifically, therefore examine only intact cells for staining interpretations.[13]

Excessive or incomplete counterstaining may compromise proper interpretation of results.

13. TROUBLESHOOTING

PROBLEM	*PROBABLE CAUSE*	*SUGGESTED ACTION*
1. No staining of any slides	1.a. Reagents not used in proper order.	1.a. Review application of reagents.
	b. Sodium azide in buffer bath.	b. Use fresh azide-free buffer.
	c. Substrate-chromogen reagent mixed incorrectly.	c. Make a fresh substrate-chromogen using the instructions included with the product.
2. Weak staining of all slides	2.a. Sections retain too much solution after wash bath.	2.a. Gently tap off excess solution before wiping around section.
	b. Substrate-chromogen mixture too old.	b. Prepare fresh substrate-chromogen solution.
	c. Slides not incubated long enough with antibodies or substrate mixture.	c. Review recommended incubation times.
3. Excessive background staining in all slides	3.a. Specimens contain high endogenous peroxidase activity.	3.a. Incubate slides with fresh hydrogen peroxide (see Section 12).
	b. Paraffin incompletely removed.	b. Use fresh xylene or toluene baths. If several slides are stained simultaneously, the second xylene bath should contain fresh xylene.

c. Slides not properly rinsed.	c. Use fresh solutions in buffer baths and wash bottles.
d. Faster than normal substrate reaction due to e.g. excessive room temperature.	d. Use shorter incubation time with substrate-chromogen solution.
e. Sections dried during staining procedure.	e. Use humidity chamber. Wipe only three to four slides at a time before applying reagent.
f. Nonspecific binding of reagents to tissue section.	f. Apply a blocking solution containing an irrelevant protein (see Section 12).
g. Primary antibody too concentrated.	g. Use higher dilution of the primary antibody.

Note: If the problem cannot be attributed to any of the above causes, or if the suggested corrective action fails to resolve the problem, please call the DAKO Technical Services Department for further assistance at 800/424-0021.

Additional information on staining techniques and specimen preparation can also be found in the "Handbook-Immunochemical Staining Methods,"[22] available from DAKO Corporation, "Atlas of Immunohistology,"[23] and "Immunoperoxidase Techniques, A Practical Approach to Tumor Diagnosis."[24]

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DAKO® Liquid DAB +
Large Volume
Substrate-Chromogen System
Code No.: K 3468

INTENDED USE

This substrate-chromogen system is a high sensitivity DAB system suitable for use in peroxidase-based immunohistochemical (IHC) and *in situ* hybridization (ISH) staining methods. Upon oxidation, DAB forms a brown end product at the site of the target antigen or nucleic acid.[1]

FOR LABORATORY USE.

REAGENTS

The following materials, sufficient for preparation of 110 mL of substrate-chromogen solution, are included. This volume is sufficient for staining a minimum of 500 specimens.

Quantity	*Description*
1 x 5 mL	DAB Chromogen: 3,3'-diaminobenzidine in chromogen solution.
1 x 110 mL	Buffered Substrate: Imidazole-HCl buffer pH 7.5 containing hydrogen peroxide and an anti-microbial agent.

PRECAUTIONS

1. The DAB Chromogen and Buffered Substrate are sensitive to contamination from a variety of oxidizing agents such as metals, bacteria, dust and commonly used laboratory glassware. To avoid contamination and premature expiration, avoid contact of these reagents with any potential source of contamination.
2. To avoid contamination, never pipette DAB Chromogen or Buffered Substrate directly from bottles. Pour out required amounts into clean containers and pipette from them. Do not return excess solutions to primary storage containers.
3. DAB has been demonstrated to be a potential carcinogen and skin contact should be avoided. If skin contact does occur, flush with copious amounts of water.
4. Handle the DAB Chromogen solution with care; use of personal protective equipment is recommended.
5. Do not store the DAB components or perform staining in strong light, such as direct sunlight.
6. Refer to the Material Saftey Data Sheet (MSDS) for additional information.

REAGENT PREPARATION

Add 1 drop (or 20 µl) of the DAB Chromogen per mL of Buffered Substrate. Use the provided graduated tube to measure the amount of Buffered Substrate needed. Mix well and apply solution using the provided transfer pipette. After use, rinse graduated test tube and pipette thoroughly with distilled water. Unused working DAB solution is stable for up to two weeks if stored at 2-8°C. If precipitate forms, mix well before using.

PROCEDURE

For IHC and ISH staining, following incubation with the HRP reagent, place specimens in buffer bath. Tap off excess buffer and carefully wipe slide around specimen.

1. Cover specimen with the DAB solution. Incubate for 5-30 minutes. If desired, the incubation with the DAB Substrate-Chromogen can be carried out at higher temperatures than room temperature.
2. Rinse gently with distilled water.
3. Counterstain, if desired.
4. Coverslip with aqueous-based or permanent mounting media.

For the capillary gap slide method, please observe the following recommendations:

1. There is no need to add detergents to this substrate-chromogen system to reduce surface tension.
2. Draw up the DAB Substrate-Chromogen for 20 to 30 seconds and blot the solution at least twice before incubating for 5-30 minutes.

RESULTS

In IHC and ISH procedures, the DAB Substrate-Chromogen yields a brown reaction end-product at the site of the target antigen or nucleic acid.

For proper interpretation, positive and negative controls should accompany each staining run. Positive controls serve as indicators that specimen processing and handling were carried out correctly. Negative controls are useful for assessing non-specific staining. Non-specific staining, if present, is rather diffuse in appearance.

LIMITATIONS

Endogenous peroxidase or pseudoperoxidase activity, found in hemoproteins such as hemoglobin, myoglobin, cytochrome, and catalase, as well as in eosinophils may yield false-positive results.[2,3] In formalin-fixed tissue this activity can be inhibited by incubating the tissue in 3% hydrogen peroxide for five minutes prior to the application of primary antibody or probe. Blood, bone marrow smears and frozen tissue sections can be treated with DAKO® Peroxidase Blocking Reagent (Code No. S 2001). However, this procedure does not abolish the reddish-brown pigment of hemoproteins. A solution of methanol-hydrogen peroxide can also be used, however, some antigens may become denatured with this procedure.

STORAGE

Store DAB Substrate-Chromogen system in original container at 2-8°C.

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Specifications

INSTRUCTIONS
LARGE VOLUME DAKO ENVISION® SYSTEM, PEROXIDASE (AEC)
UNIVERSAL
K1393

1. INTENDED USE

FOR LABORATORY USE.

These instructions apply to the **LARGE VOLUME DAKO ENVISION® SYSTEM, PEROXIDASE** (DAKO Envision® System, HRP).

This kit is intended for use with primary antibodies from **RABBIT** or **MOUSE** supplied by the user for the qualitative identification of antigens by light microscopy in normal and pathological paraffin-embedded tissues, cryostat tissues and cell preparations. Tissues processed in a variety of fixatives including ethanol, B-5, Bouin's, zinc formalin, and neutral buffered formalin may be used.

2. SUMMARY AND EXPLANATION

Immunoenzymatic/immunohistochemical (IHC) staining techniques allow for the visualization of tissue (cell) antigens. These techniques are based on the immunoreactivity of antibodies and the chemical properties of enzymes or enzyme complexes which react with colorless substrate-chromogens to produce a colored end-product. Initial immunoenzymatic stains utilized the direct method which conjugated enzymes directly to an antibody with known antigenic specificity (primary antibody). Although this technique lacked the sensitivity of later methods, it allowed for the visualization of tissue antigens using a standard light microscope.

The sensitivity of IHC stains was significantly improved with the development of an indirect technique. In this method, enzyme-labelled secondary antibodies react with the antigen-bound primary antibody. A further increase in sensitivity over the indirect technique was achieved with the introduction of the peroxidase-antiperoxidase (PAP) enzyme complex.[1] In this method the secondary antibody serves as a linking antibody between the primary antibody and the PAP complex.[1] Subsequent developments in IHC exploited the strong affinity of avidin for biotin and resulted in the avidin-biotin complex (ABC) method of Hsu et al.[2] This technique employs an enzyme labelled avidin-biotin complex which is mixed prior to use and forms a complex with a biotinylated secondary antibody. The ABC method increased sensitivity when compared to the PAP method.

IHC was further improved with the labelled streptavidin biotin (LSAB) method which is based on a modified labelled avidin-biotin (LAB) technique. The LSAB method utilizes a biotinylated secondary antibody to form a complex with peroxidase-conjugated streptavidin molecules.[3,4] In comparison to the ABC method, the LAB and LSAB methods have been reported to be eight times more sensitive.[5-7]

The Large Volume DAKO Envision® System, HRP is a two-step IHC staining technique which allows the user to choose a short or a long protocol depending on the sensitivity required. This system is based on an HRP labelled polymer which is conjugated with secondary antibodies. All reagents in the Large Volume DAKO Envision® System, HRP with AEC substrate are ready-to-use. Protocol #1 of the Large Volume DAKO Envision® System, HRP is intended to be used with the DAKO® N-series Primary Antibodies

to achieve optimal sensitivity. When compared to the LSAB method, Protocol #1 is equivalent or better in sensitivity. Protocol #2 is an extremely sensitive method and, as a result, optimal dilutions of the primary antibody are up to 20 times higher than those used for the traditional PAP technique, and several-fold greater than those used for the traditional ABC or LSAB methods. Protocol #2 offers an enhanced signal generating system for the detection of antigens present in low concentrations or for low titer primary antibodies.

Primary antibodies produced in either rabbit or mouse react equally well with the labelled polymer. The interpretation of any positive staining or its absence should be complemented by morphological and histological studies with proper controls.

3. PRINCIPLES OF PROCEDURE

Protocols #1 and #2 require the same number of steps although incubation times of the primary antibody and the labelled polymer vary. Both protocols quench any endogenous peroxidase activity by incubating the specimen for five minutes with DAKO's Peroxidase Blocking Reagent. The specimen is then incubated with an appropriately characterized and diluted rabbit or mouse primary antibody, followed by the incubation with the labelled polymer. Protocol #1 requires two sequential 10-minute incubations and Protocol #2 requires two sequential 30-minute incubations. It should be noted that for antibodies requiring enzyme digestion or target retrieval, it may be necessary to increase incubation times of the primary antibody and labelled polymer by 5 to 10 minutes. Staining is completed by a 5- to 10-minute incubation with 3-amino-9-ethylcarbazole (AEC) substrate-chromogen which results in a red-colored precipitate at the antigen site. (AEC is a potential carcinogen, see "Precautions" Section 5.)

4. REAGENTS

The following materials, sufficient for at least 500-800 tissue sections, are included in this kit:

Bottle No.	*Qty.*	*Description*
1	1x110ml	**PEROXIDASE BLOCKING REAGENT:** 0.03% hydrogen peroxide containing sodium azide.
3	1x110 ml	**LABELLED POLYMER:** Peroxidase labelled polymer conjugated to goat anti-rabbit and goat anti-mouse immunoglobulins in Tris-HCl buffer containing carrier protein and an anti-microbial agent.
4	1x110 ml	**SUBSTRATE,** AEC Chromogen: 3-amino-9-ethylcarbazole in N,N-dimethylformamide (DMF) and acetate buffer, pH 5.0, containing hydrogen peroxide, stabilizers, enhancers and an anti-microbial agent.

MATERIALS REQUIRED BUT NOT SUPPLIED

Absorbent wipes
Ammonium hydroxide, 15M diluted to 37mM
Control tissue, positive and negative
Counterstain; aqueous based, such as Mayer's hematoxylin (see Section 6.D.)
Coverslips
Distilled water
Ethanol, absolute and 95%
Light microscope (20x-800x)
Mounting media, such as DAKO Glycergel® Mounting Medium (Code No. C0563)
Primary antibodies and negative control reagent
Slides, Poly-L-Lysine coated or DAKO® Silanized Slides (see Section 8.A.6.)

Staining jars or baths
Timer (capable of 3-40 minute intervals)
Wash bottles
Wash Buffer Solution (see Section 6.A.)
Xylene, toluene or xylene substitutes

5. PRECAUTIONS

5.A. Product Specific

1. FOR LABORATORY USE.

2. The sodium azide (NaN_3) used as a preservative in this kit is toxic if ingested. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal, flush with large volumes of water to prevent azide build-up in plumbing.[8,9]

3. 3-amino-9-ethylcarbazole (AEC) may be carcinogenic. This solution may cause irritation upon skin contact. Avoid skin contact. If skin contact occurs, flush affected area with water. Waste containing AEC should be disposed of according to local, state and federal regulations.

4. N,N-dimethylformamide (DMF), used as a solvent for AEC, is a combustible compound. Use away from open flame. Avoid ingestion, inhalation or skin contact. Formamide is classified as a teratogen and pregnant workers should keep exposure to a minimum. If contact with skin occurs, wash thoroughly with soap and water.

5. Do not use reagents beyond expiration date for prescribed storage method. If reagents are stored under any conditions other than those specified in the product insert, they must be validated by the user.

6. Do not substitute reagents from other lot number or from kits of other manufacturers.

7. Enzymes and substrate-chromogens may be affected adversely if exposed to excessive light levels. Do not store kit components or perform staining in strong light, such as direct sunlight.

8. Incubation times or temperatures other than those specified may give erroneous results; any such changes must be validated by the user.

5.B. General

1. Specimens, before and after fixation, and all materials exposed to them, should be handled as if capable of transmitting infection and disposed of with proper precautions.[10] Wear disposable gloves when handling tissues.

2. Wear disposable gloves during staining procedures.

3. Inhalation or ingestion of xylene or the highly allergenic fixative formaldehyde is harmful. Prepare in fume hood. If swallowed, induce vomiting. If skin or eye contact occurs, wash thoroughly with water.

4. Organic reagents are flammable. Do not use near open flame.

5. Never pipette reagents by mouth and avoid their contact with skin and mucous membranes.

6. Avoid microbial contamination of reagents or incorrect results may occur.

7. Avoid splashing of reagents or generation of aerosols.

8. Avoid physical contact and inhalation of 15M ammonium hydroxide. Prepare in fume hood. If skin or eye contact occur, wash thoroughly with water.

6. REAGENT PREPARATION

It is convenient to prepare the following reagents prior to staining.

6.A. Wash Buffer Solution

0.02M Phosphate Buffered Saline (DAKO® PBS, Code No. S3024), 0.05M Tris-HCl buffer or 0.05M Tris Buffered Saline (DAKO® TBS, Code No. S3001), pH 7.2-7.6, **without sodium azide**, are suitable wash and rinse buffer solutions. Wash buffer solutions containing sodium azide inactivate enzyme-labelled complexes and result in negative staining. Store unused buffer at 2-80C. Discard buffer if cloudy in appearance.

Distilled water may be used for rinsing the peroxidase blocking reagent, substrate and counterstain.

6.B. Primary Antibody

A wide range of DAKO® N-series Primary Antibodies and Negative Control Reagents are available for use with Protocol #1 of the Large Volume DAKO Envision® System, HRP. Concentrated antibodies are also available from DAKO; however, optimal dilutions for Protocol #1 and Protocol #2 must be determined experimentally by the user. Due to the high sensitivity of Protocol #2 of the Large Volume DAKO Envision® System, HRP primary antibody dilutions may range from five- to twenty-fold greater than those used in traditional IHC methods. Dilutions should be prepared using 0.05M Tris-HCl buffer, pH 7.2-7.6, containing 1% bovine serum albumin (DAKO® Antibody Diluent, Code No. S0809). For most primary antibodies used with this kit, an incubation time of 10 minutes for Protocol #1 and 30 minutes for Protocol #2 is sufficient.

6.C. Negative Control Reagent

Ideally, a negative control reagent contains an antibody which exhibits no specific reactivity with human tissues or normal/nonimmune serum in the same matrix/solution as the diluted primary antibody. The negative control reagent should be the same subclass and animal species as the primary antibody, diluted to the same immunoglobulin or protein concentration as the diluted primary antibody using the same diluent. The incubation period for the negative control reagent should correspond to the primary antibody.

When using DAKO® N-series Primary Antibodies optimized for use in Protocol #1 of Large Volume DAKO Envision® System, HRP, use the provided negative control reagent. A negative control reagent should also be used with Protocol #2 of the Large Volume DAKO Envision® System, HRP. For specific information regarding negative control reagent preparation, refer to Quality Control, Section 10.C.

6.D. Counterstain

The colored end-product of the staining reaction is alcohol soluble and should only be used with aqueous-based counterstains such as Mayer's hematoxylin. Follow counterstaining of hematoxylin with a thorough rinse in distilled water, then immerse tissue slides into a bath of 37mM ammonia (see Section 9.B. Step 5) or similar bluing agent. Thirty-seven millimolar ammonia water is prepared by mixing 2.5 ml of 15M (concentrated) ammonium hydroxide with 1 liter of water.

Unused 37mM ammonia may be stored at room temperature (20-25/C) in a tightly capped bottle for up to 12 months.

Consult manufacturers' guidelines for alternative counterstaining procedures.

6.E. Mounting Media

DAKO Glycergel® Mounting Medium (Code No. C0563) is recommended for aqueous mounting. This reagent must be heated to at least 50/C just prior to use.

7. STORAGE AND HANDLING

Reagents of the Large Volume DAKO Envision® System, HRP are to be stored at 2-8°C. Do not freeze.

Large Volume DAKO Envision® Systems are suitable for use 12 months from the date of manufacture when stored at 2-8°C. Do not use after expiration printed on reagent vials and kit label.

Alteration in the appearance of any reagent, such as precipitation, may indicate instability or deterioration. In such cases, the reagent(s) is (are) not to be used.

8. SPECIMEN PREPARATION

Prior to IHC staining, tissues must be fixed and processed. Fixation prevents autolysis and putrefaction of excised tissues, preserves antigenicity, enhances the refractive index of tissue constituents and increases the resistance of cellular elements to tissue processing. Tissue processing includes dehydration, clearing of dehydrating agents, infiltration of embedding media, embedding and sectioning of tissues. The most common fixatives for IHC tissue preparations are discussed below.

These are guidelines only. Optimal procedures must be determined and verified by the user. (For specific information regarding tissue fixation and processing, see references 11 and 12.)

8.A. Paraffin-Embedded Tissue

8.A.1. *General Comments*

Although 10% neutral phosphate-buffered formalin is the most common fixative, the Large Volume DAKO Envision® System, HRP has been successfully used with tissues processed in a variety of fixatives. Consequently, the choice of fixative is dependent on the limitations of the primary antibody and the user's institutional or laboratory constraints.

Survival of tissue antigens for immunological staining may depend on the type and concentration of fixative, on fixation time, and on the size of the tissue specimen to be fixed.[13] It is important to maintain optimal, standardized fixation conditions whenever possible in order to obtain reproducible staining. Where possible, use of thinner specimens coupled with shorter fixation times is recommended. Prolonged exposure to fixatives may result in the masking of antigens and contribute to reduced staining. Zenker's fluid, B-5 and Bouin's have often been recommended as alternative fixatives for the preservation of tissue antigens sensitive to routine formalin fixation (10% neutral buffered formalin).[14,15]

See references 11 and 12, the primary antibody specification sheet(s) and the protocol(s) supplied with the fixing reagent(s) for additional information regarding tissue fixation.

8.A.2. *Tissue Fixation in Formaldehyde-Based Solution (Neutral Buffered Formalin and Bouin's)*

Most formaldehyde-based fixatives contain 10% formalin, a neutral salt, to maintain tonicity and a buffering system to maintain pH. These fixatives are well tolerated by tissues and exhibit good histological penetration. However, shrinkage or distortions may occur in poorly fixed and embedded tissue specimens. Fix small blocks of tissue (10 x 10 x 3mm) in 5-10 ml of neutral buffered formalin per block for up to 24 hours.

Bouin's solution is an alternative formaldehyde-based fixative which contains picric acid and is suitable for use on all tissues except kidney. Specimens may be fixed from 1-12 hours depending on tissue thickness. Excessively fixed tissues become brittle and adversely affect the appearance and quantity of lipids. Complete fixation with a 70% ethanol wash to precipitate soluble picrates prior to aqueous washes.

8.A.3. *Mercuric-Chloride Containing Fixatives (B-5 and Zenker's)*

Mercuric-chloride fixatives, such as B5 and Zenker's, frequently include a neutral salt to maintain tonicity and may be mixed with other fixatives. In general, mercuric-chloride fixatives are poor histological penetrators and are not well tolerated by tissue specimens. Consequently, small tissue blocks (7 x 7 x 2.5mm) and short fixation periods (1-6 hours for B5, and 2-15 hours for Zenker's) are recommended. After fixation, the tissue block(s) should be rinsed well with water and placed in 70% ethanol for wet storage or until tissue processing can be completed. Conclude fixation with tissue processing and paraffin embedding (see Section 8.A.5.).

Prior to immunostaining, clear tissue sections of mercury deposits using an iodine/ethanol/sodium thiosulfate solution.[16] Exercise the necessary precautions when handling reagents containing mercury compounds.

8.A.4. *Tissue Fixation in Ethanol*

Ethanol is not widely employed as a fixative for routine histological techniques due to its poor penetrating ability. However, small pieces of tissue are fixed rapidly and show good cytological preservation. Fix tissue blocks (5 x 5 x 2mm) in absolute alcohol for 48 hours at room temperature (200- 250C) followed by two 1-hour baths in fresh xylene and two 1-hour baths in liquid paraffin. Follow paraffin infiltration with embedding.

8.A.5. *Processing and Paraffin Embedding*

After fixation, processing may be completed using an automatic tissue processor. Tissues are dehydrated using graded alcohols, cleared with xylene or xylene substitute, and infiltrated with paraffin wax. The tissue is subsequently embedded with paraffin wax in molds or cassettes which facilitate tissue sectioning. To minimize denaturing of antigens, do not expose tissues to temperatures in excess of 600C during processing.

Tissue blocks may be stored or sectioned on completion of embedding. Properly fixed and paraffin-embedded tissues will keep indefinitely if stored in a cool place.

8.A.6. *Adherence of Paraffin-Embedded Tissue Sections to Microscope Slides*

Collect sectioned tissues from paraffin-embedded blocks on clean glass slides. Dehydrate in an oven for one to two hours at 600C or less. For increased adhesion of tissue sections during IHC staining procedures, use of poly-L-lysine coated slides or DAKO® Silanized Slides (Code No. S3003) is suggested. Coated slides are strongly recommended for staining procedures requiring proteolytic digestion or target retrieval.

Slides with paraffin-embedded tissue sections can be kept indefinitely if stored in a cool place.

8.A.7. *Deparaffinization and Rehydration*

Prior to staining, tissue slides must be deparaffinized to remove embedding media and rehydrated. Avoid incomplete removal of paraffin. Residual embedding media will result in increased nonspecific staining.

1. Place slides in a xylene bath and incubate for 5 (±1) minutes. Change baths and repeat once.

2. Tap off excess liquid and place slides in absolute ethanol for 3 (±1) minutes. Change baths and repeat once.

3. Tap off excess liquid and place slides in 95% ethanol for 3 (±1) minutes. Change baths and repeat once.

4. Tap off excess liquid and place slides in distilled or deionized water for a minimum of 30 seconds. Unless proteolytic digestion is required, commence staining procedure as outlined in Section 9.B.

Xylene and alcohol solutions should be changed after 40 slides. Toluene or xylene substitutes such as Histoclear may be used in place of xylene.

If necessary, rehydrated tissues may be kept in buffer solution at 2-80C for up to 18 hours prior to use. Allow tissues to come to room temperature (200-250C) before staining.

8.A.8. *Proteolytic Digestion and Target Retrieval*

Formaldehyde is known to induce conformational changes in the antigen molecules by forming intermolecular cross-linkages. Excessive formalin fixation can mask antigenic sites and diminish specific staining. However, these sites may be revealed with proteolytic digestion or target retrieval of tissue slides prior to immunostaining. To determine if either of these pretreatments of tissues is warranted, see the specification sheet provided with each primary antibody.

Deparaffinized and rehydrated tissue sections can be digested for six minutes at room temperature (20°-25°C) in a solution containing 0.025% Protease type XXIV and 0.025% $CaCl_2$ in a Tris-HCl buffer, pH 7.2-7.6. Rinse thoroughly with distilled water and continue with the staining procedure as outlined in Section 9.B. Other proteolytic enzymes, such as proteinase K (DAKO® Proteinase K, Code No.S3020), Pepsin (DAKO® Pepsin, Code No. S3002), pronase or trypsin can also be used.

Target retrieval prior to IHC staining procedures results in an increase in staining intensity with many primary antibodies. For some antibodies, this procedure is required. The retrieval procedure involves immersion of tissue sections mounted on slides in solution (DAKO® Target Retrieval Solution, Code No. S1700) and heating prior to IHC staining. Rinse thoroughly with 0.05M Tris-HCl and continue with the staining procedure outlined in Section 9.B.

***NOTE:* Overdigestion may result in nonspecific staining and/or unacceptable morphology.**

8.B. Frozen Tissue

Frozen sections should be cut from snap-frozen tissue blocks (approximately 1.0 x 1.0 x 0.5 cm) and air-dried for 2-24 hours. Dried sections can be fixed in room temperature (200 - 250C) acetone for 10 minutes or in buffered formyl-acetone for 30 seconds. Allow sections to air-dry until completely dehydrated. Proceed with immunostaining or wrap slides in aluminum foil and store at -200C or lower for up to three to six months. Equilibrate wrapped, frozen sections to room temperature prior to use.

If sections are too thick (greater than 4 Tm), incorrectly fixed, or unevenly dried, artifacts may result and interfere with interpretation of staining. This includes rupturing of cell membranes and chromatolysis. Nuclei may appear swollen and uniformly blue when counterstained with hematoxylin.

8.C. Other Specimens

The Large Volume DAKO Envision® System, HRP may also be used for staining antigens in bone sections, bone marrow, blood smears, cytospins and imprints. Smears may be air-dried for 2-24 hours and processed for immediate staining or wrapped in aluminum foil and stored at -200C or lower for up to three to six months. Air-dried or thawed smears may be fixed for 90 seconds in acetone-methanol (1:1). Fixation in acetone or acetone-methanol-formalin (10:10:1) is also acceptable.

Osseous tissues must be decalcified prior to sectioning and processing to facilitate tissue cutting and prevent damage to microtome blades.[11]

9. STAINING PROCEDURE

9.A. Procedural Notes

The user should read these instructions carefully and become familiar with the kit contents prior to use. Condensed instructions appear on the inside cover of the kit box and are keyed to the colors and numbers of each kit reagent. See Section 5 for precautions.

The Large Volume DAKO Envision® System, HRP offers the user a choice of two protocols depending on the sensitivity desired. Protocol #1 is intended to be used with DAKO® N-series Primary Antibodies. Protocol #2 is a more sensitive method and optimal dilutions of the primary antibody are several-fold greater than those used for the traditional ABC or LSAB methods. Protocol #1 and Protocol #2 use the same bottled reagents provided in the Large Volume DAKO Envision® System. The only difference is an increase in incubation time of each step for Protocol #2.

The reagents and instructions supplied in this kit have been designed for optimal performance. Further dilution of the kit reagents or alteration of incubation times or temperatures may give erroneous results.

Bottle 1 and Bottle 3 should be equilibrated to room temperature (200- 250C) prior to immunostaining. Likewise, all incubations should be performed at room temperature. **For convenience the AEC substrate may be used immediately after removal from the refrigerator and does not have to be brought to room temperature before use. After use, return to 2-8/C storage. Storage at temperature above 8/C adversely affects the stability of the AEC substrate-chromogen.**

Do not allow tissue sections to dry during the staining procedure. Dried tissue sections may display increased nonspecific staining. Cover slides exposed to drafts. If prolonged incubations are used, place tissues in a humid environment.

The sensitivity of the Large Volume DAKO Envision® System can be further increased by lengthening the incubation times of step 2 and 3 five to ten minutes.

9.B. Staining Protocol

STEP 1: PEROXIDASE BLOCK REAGENT

Tap off excess buffer. Using a lintless tissue (such as Kimwipe or gauze pad), carefully wipe around the specimen to remove any remaining liquid and to keep reagent within the prescribed area.

Apply enough Peroxidase Blocking Reagent from Bottle 1 to cover specimen.

Incubate 5 (± 1) minutes.

Rinse gently with distilled water or buffer solution from a wash bottle (do not focus flow directly on tissue) and place in fresh buffer bath.

STEP 2: PRIMARY ANTIBODY OR NEGATIVE CONTROL REAGENT

Tap off excess buffer and wipe slides as before.

Apply enough primary antibody or negative control reagent to cover specimen.

Protocol #1 - incubate 10 (±1) minutes
Protocol #2 - incubate 30 (±1) minutes

Rinse gently with buffer solution from a wash bottle (do not focus flow directly on tissue) and place in fresh buffer bath.

If the staining procedure must be interrupted, slides may be kept in a buffer bath following incubation of the primary antibody (Step2) for up to one hour at room temperature (20/ - 25/C) without affecting the staining performance.

STEP 3: PEROXIDASE LABELLED POLYMER

Tap off excess buffer and wipe slides as before.

Apply enough drops from Bottle 3 (Labelled Polymer) to cover specimen.

Protocol #1 - Incubate 10 (±1) minutes.
Protocol #2 - Incubate 30 (±1) minutes.

Rinse slides as in Step 2.

STEP 4: SUBSTRATE-CHROMOGEN

Wipe slides as before.

Apply enough of the ready-to-use substrate-chromogen solution to cover specimen.

Incubate for 5-10 minutes.

Rinse gently with distilled water from a wash bottle (do not focus flow directly on tissue). Collect substrate-chromogen waste in a hazardous materials container for proper disposal.

STEP 5: HEMATOXYLIN COUNTERSTAIN (optional)

Immerse slides in a bath of aqueous hematoxylin. Incubate for 2-5 minutes, depending on the strength of hematoxylin used.

Rinse gently in a distilled water bath.

Dip slides 10 times into a bath of 37mM ammonia or similar bluing agent. (See Section 6.D.)

Rinse slides in a bath of distilled or deionized water for 2-5 minutes.

STEP 6: MOUNTING

Specimens may be mounted and coverslipped with an aqueous-based mounting medium such as DAKO Glycergel® Mounting Medium (Code No. C0563).

NOTE: The AEC reaction product is soluble in organic solvents and is therefore not compatible with toluene- or xylene-based permanent mounting media.

NOTE: Slides may be read when convenient. However, some fading may occur if slides are exposed to strong light over a period of one week. To minimize fading, store slides in the dark at room temperature (200 - 250C).

10. QUALITY CONTROL

Differences in tissue processing and technical procedures in the user's laboratory may produce significant variability in results, necessitating regular performance of in-house controls in addition to the following procedures. See the quality control guidelines of the College of American Pathologists (CAP) Certification Program for Immunohistochemistry and references 17 through 19 for additional information. Refer to the specification sheet of each primary antibody used for details regarding sensitivity and immunoreactivity.

10.A. Positive Control Tissue

Controls should be fresh surgical specimens or autopsy tissues fixed, processed and embedded in the same manner as the test sample(s). Positive control tissues are indicative of correctly prepared tissues and proper staining techniques. One positive control tissue for each set of test conditions should be included in each staining run.

Commercially available tissue slides (such as DAKO® Control Slides, Code Nos. T1064 and T1065), or specimens processed differently from the test sample(s) validate reagent performance only, and do not verify tissue preparation.

Known positive control tissues should only be utilized for monitoring the correct performance of processed tissues and test reagents. If the positive control tissues fail to demonstrate positive staining, results with the test specimens should be considered invalid.

10.B. Negative Control Tissue

One negative control tissue fixed, processed and embedded in a manner identical to the test sample(s) should be stained with each primary antibody used in each staining run to verify the specificity of each primary antibody. Additionally, the variety of different cell types present in most tissue sections frequently offers internal negative control sites, but this should be verified by the user.

If specific staining occurs in the negative control tissue, results with the test specimen should be considered invalid.

10.C. Negative Control Reagent

Use a negative control reagent with each specimen to evaluate nonspecific staining and allow better interpretation of specific staining at the antigen site. Ideally, a negative control reagent contains an antibody which exhibits no specific reactivity with human tissues (non-human reactive) in the same matrix/solution as the diluted primary antibody. The non-human reactive antibody should be the same subclass and animal species as the primary antibody, diluted to the same immunoglobulin or protein concentration as the diluted primary antibody. Normal/nonimmune serum from the same species as the primary antibody, at a protein concentration equivalent to the diluted primary antibody in the same matrix/solution may be suitable for use depending on the type of primary antibody used. Refer to the specification sheet of each primary antibody and the example below for specific recommendations. Diluent alone may be used as a less desirable alternative to the previously described negative control reagents. The incubation period for the negative control reagent should correspond to the primary antibody.

Examples of Negative Control Reagents for Concentrated Antibodies:

Primary Antibody Type	Suggested Negative Control Reagent
monoclonal mouse antibody, produced in ascites	non-human reactive monoclonal mouse antibody, produced in ascites; or normal/nonimmune mouse serum, Code No. X0910
monoclonal mouse antibody, produced in tissue culture, subclass IgG_1	non-human reactive monoclonal mouse antibody produced in tissue culture, subclass IgG_1, DAKO Code No. X0931; or fetal calf serum*
monoclonal mouse antibody produced in tissue culture, subclass IgG_2a	non-human reactive monoclonal mouse antibody produced in tissue culture, subclass IgG_2a, DAKO Code No. X0943; or fetal calf serum*
monoclonal mouse antibody produced in tissue culture, subclass IgG_2b	non-human reactive monoclonal mouse antibody produced in tissue culture, subclass IgG_2b, DAKO Code No. X0944; or fetal calf serum*
monoclonal mouse antibody produced in tissue culture, subclass IgM	non-human reactive monoclonal mouse antibody produced in tissue culture, subclass IgM, DAKO Code No. X0942; or fetal calf serum*
polyclonal rabbit antibody, immunoglobulin fraction	normal/nonimmune rabbit serum, immunoglobulin fraction, DAKO Code No. X0903
polyclonal rabbit antibody, whole serum	normal/nonimmune whole rabbit serum, DAKO Code No. X0902

* fetal calf serum is also suitable for use as it is retained in the primary antibody after processing

If panels of several antibodies are used on serial tissue sections, the negatively staining areas of one slide may serve as a negative/nonspecific binding background control for other antibodies.

NOTE: When using DAKO® N-series Primary Antibodies optimized for use with Protocol #1 of Large Volume DAKO Envision® System, HRP use the provided negative control reagent. A negative control reagent should also be used with Protocol #2 of the Large Volume DAKO Envision® System, HRP, using the above chart as a guideline to match the optimized primary antibody dilution.

To differentiate endogenous enzyme activity or nonspecific binding of enzymes from specific immunoreactivity, additional test tissues may be stained exclusively with prepared substrate AEC chromogen.

For remedial solutions to aberrant test results refer to Troubleshooting, Section 13.

10.D. Assay Verification

Prior to initial use of an antibody or staining system in a procedure, the user should verify the antibody's specificity by testing it on a series of in-house tissues with known immunohistochemical performance characteristics. Refer to the quality control procedures outlined in the above subsections A, B and C, and the Quality Control Section of specific primary antibody. Assay verification should be repeated for each new antibody lot or whenever there is a change in assay parameters.

11. INTERPRETATION OF STAINING

11.A. Positive Control Tissue

The positive control tissue should be examined first to ascertain that all reagents are functioning properly. Presence of a red-colored end-product at the site of the target antigen is indicative of positive reactivity. If the positive control tissues fail to demonstrate positive staining, results with the test specimens should be considered invalid.

11.B. Negative Control Tissue

The negative control tissue should be examined after the positive control tissue to verify the specific labelling of the target antigen by the primary antibody. The absence of specific staining in the negative control tissue confirms the lack of antibody cross-reactivity to cells/cellular components. If specific staining occurs in the negative control tissue, results with the test specimen should be considered invalid.

Nonspecific staining, if present, will be of a diffuse appearance. Sporadic light staining of connective tissue may be observed in sections from excessively formalin-fixed tissues. Use intact cells for interpretation of staining results. Necrotic or degenerated cells often stain nonspecifically.

False-positive results may be seen due to non-immunologic binding of reagents to tissue sections. In some cases the application of blocking reagent prior to incubation with the primary antibody may be useful for reducing background. A recommended blocking reagent is DAKO® Protein Block Serum-Free (Code No. X0909). Alternatively, a blocking solution can be made from normal swine serum, such as DAKO® Serum (Normal) Swine (Code No. X0901), diluted to 20% in 0.05M Tris-HCl buffer, pH 7.2-7.6.

11.C. Test Tissue

Test specimens stained with the primary antibody should be examined last. Positive staining intensity should be assessed within the context of any nonspecific background staining of the negative control reagent. The absence of a specific positive staining reaction can be interpreted as no antigen detected. If necessary, use a panel of antibodies to identify false-negative reactions.

11.D. General Comments

Precipitates may form if specimens are allowed to dry during the staining procedure. This may be apparent at the edge of the specimen. Scan tissues at 40X magnification to avoid misinterpretation of results.

Depending on the incubation length and potency of the hematoxylin used, counterstaining will result in a pale to dark blue coloration of cell nuclei. Excessive or incomplete counterstaining may compromise proper interpretation of results.

12. LIMITATIONS

Tissue staining is dependant on the proper handling and processing of tissues prior to staining. Improper fixation, freezing, thawing, washing, drying, heating, sectioning or contamination with other tissues or fluids may produce artifacts, antibody trapping, or false-negative results.

Use of old or unbuffered fixatives, or exposure of tissues to excessive heat (greater than 600C) during processing may result in decreased staining sensitivity.

Endogenous peroxidase or pseudoperoxidase activity can be found in hemoproteins such as hemoglobin, myoglobin, cytochrome, and catalase as well as in eosinophils.[20,21] This activity can be inhibited by incubating specimens with Peroxidase Blocking Reagent Bottle #1 of the Large Volume DAKO Envision® System, HRP for five minutes prior to the application of the primary antibody. Blood and bone marrow smears and frozen tissues can also be treated with this reagent. However, this procedure does not abolish the reddish-brown pigment of hemoproteins. Alternately, a solution of methanol-hydrogen peroxide can be

used. Some antigens may become denatured with this procedure.

Tissues from persons infected with hepatitis B virus and containing hepatitis B surface antigen (HBsAg) may exhibit nonspecific staining with horseradish peroxidase.[22]

Normal/nonimmune sera from the same animal source as the secondary antisera used in blocking steps may cause false-negative or false-positive results due to auto-antibodies or natural antibodies.

The reagents supplied in this kit have been optimally diluted. Further dilution may result in loss of antigen detection.

13. TROUBLESHOOTING

PROBLEM	*PROBABLE CAUSE*	*SUGGESTED ACTION*
1. No staining of any slides	1a. Reagents not used in proper order.	1a. Review application of reagents.
	1b. Sodium azide in buffer bath.	1b. Use fresh azide-free buffer.
2. Weak staining of all slides	2a. Sections retain too much solution after wash bath.	2a. Gently tap off excess solution before wiping around section.
	2b. Slides not incubated long enough with antibodies or substrate.	2b. Review recommended incubation times.
3. Excessive background staining in all slides	3a. Specimens contain high endogenous peroxidase activity.	3a. Use longer incubation time of peroxidase block, Bottle #1.
	3b. Paraffin incompletely removed.	3b. Use fresh xylene or toluene baths. If several slides are stained simultaneously, the second xylene bath should contain fresh xylene.
	3c. Slides not properly rinsed.	3c. Use fresh solutions in buffer baths and wash bottles.
	3d. Faster than normal substrate reaction due to e.g. excessive room temperature.	3d. Use shorter incubation time with substrate-chromogen solution.
	3e. Sections dried during staining procedure.	3e. Use humidity chamber. Wipe only three to four slides at a time before applying reagent.
	3f. Nonspecific binding of reagents to tissue section.	3f. Apply a blocking solution containing an irrelevant protein (see Section 11.B.).

NOTE: If the problem cannot be attributed to any of the above causes, or if the suggested corrective action fails to resolve the problem, please call the DAKO Technical Services Department for further assistance (1-800-424-0021).

Additional information on staining techniques and specimen preparation can also be found in the "Handbook-Immunochemical Staining Methods,"[12] available from DAKO Corporation, "Atlas of Immunohistology,"[23] and "Immunoperoxidase Techniques, A Practical Approach to Tumor Diagnosis."[24]

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